

**HUMAN GENE TRANSFER/THERAPY PROTOCOL**

**9212-036**

Welsh, Michael J., Howard Hughes Medical Institute and Smith, Alan E.; Genzyme Corporation; *Cystic Fibrosis Gene Therapy Using an Adenovirus Vector: In Vivo Safety and Efficacy in Nasal Epithelium.*  
Date of RAC Approval: 12-4-92

**APPLICATION TO  
NATIONAL INSTITUTES OF HEALTH  
RECOMBINANT DNA ADVISORY COMMITTEE**

**TITLE**

**CYSTIC FIBROSIS GENE THERAPY USING AN ADENOVIRUS  
VECTOR: IN VIVO SAFETY AND EFFICACY IN NASAL EPITHELIUM**

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## 1.0 INTRODUCTION

Cystic fibrosis (CF) is a common lethal, autosomal recessive disease of Caucasians (1-3) caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) (4-6). CFTR is a chloride channel which is regulated by phosphorylation and by intracellular nucleotides (7). Mutations in the CFTR gene cause a loss of function of the CFTR chloride channel and thus, contribute to the hallmark of the disease: defective electrolyte transport by affected epithelia (1,8). Although there are a wide variety of clinical manifestations, lung disease, and particularly disease of the pulmonary airways, is the major cause of morbidity and mortality. Despite current standard therapy, the median age of survival is only 26 years. Unfortunately, no available therapy treats the basic defect.

Gene therapy to deliver CFTR cDNA could correct the molecular defect; thus it would represent a major advance in treatment of CF. Because lung disease is currently the most severe clinical manifestation of the disease, the initial target cells will be those of the airway epithelia. The feasibility of gene therapy was initially demonstrated by our finding that expression of the cDNA for wild-type CFTR corrected the chloride channel defect in cultured CF airway epithelia (9). Our eventual goal is to deliver human CFTR cDNA to the airway epithelia of CF patients. For these studies, we will test a recombinant type 2 adenovirus as a vector. Adenoviruses provide an attractive vector for introduction of the cDNA, because they appear to be safe, they do not require cell division for expression, they have a natural tropism for respiratory epithelia (10), and they have been used to deliver CFTR cDNA to the lungs of cotton rats (11).

We have constructed a recombinant adenovirus vector in which the CFTR cDNA replaces the E1 region of a type 2 adenovirus, Ad2/CFTR-1. We have shown that this vector can transfer CFTR to airway epithelia in both cell culture models and in primate experiments and we have shown that it can complement the CF chloride transport defect. We have also assessed the safety of the vector system using *in vitro* cell culture models and *in vivo* experiments in hamsters and primates.

We now propose to test the feasibility of using Ad2/CFTR-1 to deliver CFTR cDNA by applying the vector to a limited region of nasal epithelium in three CF patients. We will use the nasal epithelium for these studies for several reasons. a) Nasal epithelium has morphology and function similar to that of intrapulmonary airways, and nasal epithelia manifest the CF electrolyte transport defect. b) The nasal respiratory epithelium provides several advantages in safety for the first gene therapy trials because: the amount of virus can be minimized; access to nasal epithelia is easier and carries less risk than for pulmonary epithelia; and if adverse reactions should occur, the potential consequences for the patient are reduced. c) The use of nasal epithelium will allow us to assess noninvasively the efficacy of gene replacement by measuring directly the electrical potential difference across the nasal epithelium.

The goal of this study will be to: a) assess the safety of the current recombinant adenoviral vector when applied to human airway epithelium *in vivo*; b) assess the efficacy in correcting the CF chloride transport defect *in vivo*; and c) assess the effect of dose of recombinant adenovirus on safety and efficacy.

The results of these studies will be important in the development of future studies designed to deliver CFTR cDNA to the pulmonary airways and in the design of future generations of adenovirus vector.

## 2.0 BACKGROUND AND RATIONALE

### 2.1 Morbidity and Mortality in Cystic Fibrosis

Cystic fibrosis is the most common lethal genetic disease afflicting the Caucasian population (1). Approximately one in every 2,500 infants in the United States is born with the disease. There are approximately 30,000 CF patients in the United States. It is the predominant cause of severe chronic lung disease in children and young adults. Although improvements have been made over the last 20 years in alleviating certain symptoms of the disease and delaying its progress, the underlying disease remains untreated and patients have a life expectancy of only 20 to 30 years. Statistics from the U.S. Cystic Fibrosis Foundation reveal that with intensive therapy, the prognosis has reached 75% survival to late teens, 50% survival to 26 years of age, but survival remains only 40% to the fourth decade. Individuals with CF rarely live into the fifth and sixth decades of life. In 1986, the United States CF Patient Registry revealed only 20% of patients to be older than 18 years of age.

Disease of the pulmonary airways is the major cause of morbidity and is responsible for 95% of the mortality (1,2). The first manifestation of lung disease is often a cough, followed by progressive dyspnea. Tenacious sputum becomes purulent because of colonization with *Staphylococcus* and then with *Pseudomonas*. Chronic bronchitis and bronchiectasis can be partially treated with current therapy, but the course is punctuated by increasingly frequent exacerbations of the pulmonary disease. As the disease progresses, the patient's activity is progressively limited. End-stage lung disease is heralded by increasing hypoxemia, pulmonary hypertension, and cor pulmonale.

The upper airways of the nose and sinuses are also involved by CF. Most patients with CF develop chronic sinusitis (1,2). Nasal polyps occur in 15-20% of patients and are common by the second decade of life.

Gastrointestinal problems are also frequent in CF (1,2); infants may suffer meconium ileus. Exocrine pancreatic insufficiency, which produces symptoms of malabsorption, is present in the large majority of patients with CF. Males are almost uniformly infertile and fertility is decreased in females.

### 2.2 Current Treatment of Cystic Fibrosis

To date, the primary objectives of treatment for CF have been to control infection, promote mucus clearance, and improve nutrition (1). Intensive antibiotic use and a program of postural drainage with chest percussion are the mainstays of therapy. However, as the disease progresses, frequent hospitalizations are required. Nutritional regimens include pancreatic enzymes and fat-soluble vitamins. Bronchodilators are used at times. Corticosteroids have been used to reduce inflammation, but they may produce significant adverse effects and their benefits are not certain. In extreme cases, lung transplantation is sometimes attempted (12).

Most efforts to develop new therapies for CF have focused on the pulmonary complications. Because CF mucus consists of a high concentration of DNA, derived from lysed neutrophils, one approach has been to develop recombinant human DNase (13,14). Preliminary reports suggest that aerosolized enzyme may be effective in reducing the viscosity of mucus. This could be helpful in clearing the airways of obstruction and perhaps in reducing infections. In an attempt to limit damage caused by excess of neutrophil derived elastase, protease inhibitors have been tested. Alpha-1-antitrypsin

purified from human plasma has been aerosolized to deliver enzyme activity to the lung of CF patients (15). Another approach would be the use of agents to inhibit the action of oxidants derived from neutrophils. Although biochemical parameters have been successfully measured, the long term beneficial effects of these treatments have not been established.

Using a different rationale, other investigators have attempted to use pharmacological agents to reverse the abnormally decreased chloride secretion and increased sodium absorption in CF airways (16). Defective electrolyte transport by airway epithelia is thought to alter the composition of the respiratory secretions and mucus (1,8,16). Hence, pharmacological treatments aimed at correcting the abnormalities in electrolyte transport could be beneficial. Trials are in progress with aerosolized versions of the drug amiloride; amiloride is a diuretic that inhibits sodium channels, thereby inhibiting sodium absorption. Initial results indicate that the drug is safe and suggest a slight change in the rate of disease progression, as measured by lung function tests (17,18). Nucleotides, such as ATP or UTP, stimulate purinergic receptors in the airway epithelium. As a result, they open a class of chloride channel that is different from CFTR chloride channels. *In vitro* studies indicate that ATP and UTP can stimulate chloride secretion (19). Preliminary trials to test the ability of nucleotides to stimulate secretion *in vivo*, and thereby correct the electrolyte transport abnormalities are underway.

Despite progress in therapy, CF remains a lethal disease, and no current therapy treats the basic defect.

## 2.3 The CF Gene and Gene Product

Three areas of knowledge now allow new therapies to be developed for CF: a) knowledge about the gene which encodes CFTR; b) knowledge about the function and biochemistry of the protein product, CFTR; and c) knowledge about the molecular basis of the disease, i.e., how mutations in the gene cause dysfunction of CFTR.

### 2.3.1 The gene encoding CFTR.

The gene encoding CFTR is located in region q31 of chromosome 7 (4,5). It comprises about 240 kb and contains 27 exons. The mRNA is about 6.5 kb. The gene encodes a protein named cystic fibrosis transmembrane conductance regulator (CFTR). The protein comprises 1,480 amino acids.

Based on amino acid sequence similarity and similar predicted topology, CFTR appears to belong to a family of proteins named the traffic ATPases (20) or ABC (ATP Binding Cassette) transporters (21). CFTR is predicted to contain five domains (5): two membrane spanning domains, each composed of six transmembrane segments; a unique R (regulatory) domain, which contains several consensus phosphorylation sequences; and two nucleotide binding domains, which were predicted to interact with ATP.

### 2.3.2 CFTR function.

Availability of the cDNA encoding CFTR has allowed the protein to be expressed in cells. In the first functional and biochemical studies, we expressed CFTR cDNA in primary cultures of CF airway epithelia (9,22) and Drumm and coworkers expressed CFTR in a pancreatic epithelial cell line (23). Expression of wild-type CFTR corrected the defect in cAMP-regulated chloride permeability. This result demonstrated a causal relationship between mutations in the CFTR gene and the CF phenotype. More importantly, this

experiment established the feasibility of gene therapy to reverse the effects of the abnormal gene in CF patients.

Several antibodies to CFTR have been developed and these have allowed identification of the protein (22,24-31). Moreover, we have shown that in normal human airway epithelia, the protein is located in the apical membrane (32), where it can mediate chloride transport.

Although complementation of chloride transport in CF cells did not identify the function of CFTR, data obtained since then provide compelling evidence that CFTR is a chloride channel with novel regulation (for a review see 7). The protein has been expressed in a variety of recombinant cells (25,29,33-39); in each case, cAMP-regulated chloride channels were generated. Channels produced by recombinant CFTR and those endogenous to the apical membrane of normal secretory epithelia (40-43) have the same regulatory and biophysical properties. In addition, we have shown that alteration of specific amino acid residues in CFTR alters the anion selectivity of chloride channels (35). This observation indicates that the protein itself interacts with permeating anions, thereby indicating that CFTR forms the channel pore. Most recently, CFTR has been purified, reconstituted into proteoliposomes, and fused with planar lipid bilayers (44); in a bilayer, CFTR had regulatory and biophysical properties similar to those observed in the native cell membrane.

Other experiments on the expression of CFTR in recombinant cells indicate that the CFTR chloride channel is regulated by phosphorylation by cAMP-dependent protein kinase (34,38,45,46). We showed by site-directed mutagenesis of CFTR, followed by electrophysiological studies, that the R domain acts to regulate passage of chloride through the channel. ATP is also required on the cytosolic surface to keep CFTR chloride channels open (47). Recent work indicates that ATP interacts with the nucleotide-binding domains of CFTR to control chloride channel opening and closing (48).

These data indicate that CFTR is a regulated chloride channel. They do not, however, exclude the possibility that CFTR also has other functions (49-52). Further, although CFTR is active in the apical surface of epithelial cells, it may also function at other locations within the cell (53). The possibility that CFTR might function other than as a chloride channel or at other cellular locations make gene therapy a particularly attractive therapeutic strategy.

### 2.3.3 Cystic fibrosis-associated mutations in CFTR.

The most common CF-associated mutation, accounting for about 70% of CF chromosomes, is deletion of phenylalanine at position 508 ( $\Delta F508$ ) (6,54). Phenylalanine 508 is located in the middle of the first nucleotide-binding domain. Over 100 other mutations have been discovered on CF chromosomes (for reviews, see 3,55,56).

Mutations in CFTR appear to result in a loss of chloride channel activity in one of three general ways.

- a. The mutated protein does not traffic to the apical membrane. Our recent studies (57,58) indicate that several CF-associated mutations, including the most common,  $\Delta F508$ , lead to incomplete protein processing. This inference was based on the finding that wild-type CFTR expressed in heterologous cells underwent two stages of glycosylation, a core glycosylation (endoglycosidase H sensitive) characteristic of processing in the endoplasmic reticulum, and more extensive glycosylation, characteristic of processing in the Golgi complex. In contrast, CFTR $\Delta F508$  only underwent core glycosylation. This result suggested that the mutant protein was retained in the endoplasmic reticulum, did not reach the Golgi complex, and was not delivered to the plasma membrane. We proposed that the

mutant protein was misfolded and therefore recognized by the cellular quality control mechanism located in the endoplasmic reticulum and was degraded. Early data on this hypothesis was conflicting (29-31,39). More recently, we evaluated the hypothesis in airway epithelia using a quantitative assay to detect CFTR in the apical membrane (32). CFTR was present in the apical membrane of normal airway epithelia, but none was detected in the apical membrane of CF epithelia bearing the  $\Delta F508$  mutation. Studies in the sweat gland duct epithelium have also shown that in CF patients with the  $\Delta F508$  mutation, the mutant protein is mislocalized and is probably not present in the plasma membrane (59). The conclusion that the  $\Delta F508$  mutation causes defective protein processing was also supported by our studies that tested the effect of temperature on processing of CFTR $\Delta F508$ ; a reduction in incubation temperature can "correct" the processing of some mutant proteins. We found that the processing of CFTR $\Delta F508$  reverted toward that of wild-type as the incubation temperature was reduced (60). When the processing defect was corrected, cAMP-regulated chloride channels appeared in the plasma membrane. This result also explained some earlier data, obtained from *Xenopus* oocytes (oocytes are grown at 19°C); that data suggested that CFTR containing the  $\Delta F508$  mutation was present in the plasma membrane (39).

The results of these studies indicate that the CFTR chloride channel is either missing from the apical membrane of CF cells bearing the  $\Delta F508$  mutation and some other mutations (32,57,58), or it is present at much reduced levels. Thus the results explain the lack of apical chloride permeability in most CF epithelia. Unfortunately, because the protein is mislocalized, therapeutic attempts designed to alter the function of mutant CFTR are not expected to be successful for the majority of patients. On the other hand, pharmacological attempts to relocate mutant CFTR could be attempted.

b. The mutated protein may have abnormally reduced chloride channel function. Some CF-associated mutations generate proteins that appear to be processed correctly and hence are presumably correctly located in the plasma membrane (57). Nevertheless, they do not function normally (58). Examples include: the G551D mutation, which has no detectable function in halide efflux assays (58); the G1349D mutation, which produces a chloride channel that has a markedly reduced probability of being open (48,58); and the S1255P mutation, which has a significantly reduced stimulation by intracellular ATP (48). It is also possible that some mutations could lead to a defect in both intracellular trafficking and function.

c. Some mutations fail to produce a complete protein. Some mutations produce frame shifts, splicing mutations, or premature termination of translation (3,55,56). Such mutations would be expected to fail to generate a complete protein. Recent data suggest that some mutations that encode a premature stop codon lead to markedly reduced levels of mRNA and thus presumably little or no protein (61).

### 3.0 CONSIDERATIONS FOR GENE THERAPY OF CF

Gene therapy of CF presents several considerations that are different from those addressed in most previous proposals submitted to the RAC.

#### 3.1 Target Tissue

Because 95% of CF patients die of lung disease, the lung will be the main target for gene therapy. The hallmark abnormality of the disease is defective electrolyte transport by the epithelial cells that line the airways. Numerous investigators (reviewed in 8) have

observed: a) a complete loss of cAMP-mediated transepithelial chloride secretion, and b) a two to three fold increase in the rate of  $\text{Na}^+$  absorption. cAMP-stimulated chloride secretion requires a chloride channel in the apical membrane (62). The discovery that CFTR is a phosphorylation-regulated chloride channel and that the properties of the CFTR chloride channel are the same as those of the chloride channels in the apical membrane, indicate that CFTR itself mediates transepithelial chloride secretion. This conclusion was supported by studies localizing CFTR in lung tissue: CFTR is located in the apical membrane of airway epithelia (32) and has been reported to be present in the submucosal glands (63,64). As a consequence of loss of CFTR function, there is a loss of cAMP-regulated transepithelial chloride secretion. At this time it is uncertain how dysfunction of CFTR produces an increase in the rate of  $\text{Na}^+$  absorption. However, it is thought that the defective chloride secretion and increased  $\text{Na}^+$  absorption lead to an alteration of the respiratory tract fluid and hence, to defective mucociliary clearance, a normal pulmonary defense mechanism. As a result, clearance of inhaled material from the lung is impaired and repeated infections ensue. Although the presumed abnormalities in respiratory tract fluid and mucociliary clearance provide a plausible explanation for the disease, a precise understanding of the pathogenesis is still lacking.

Correction of the genetic defect in the airway epithelial cells is likely to reverse the CF pulmonary phenotype. The identity of the specific cells in the airway epithelium that express CFTR cannot be accurately determined by immunocytochemical means, because of the low abundance of protein. However, functional studies suggest that the ciliated epithelial cells and perhaps nonciliated cells of the surface epithelium are among the main cell types involved in electrolyte transport (62). Thus in practical terms, the present preferred target cell for gene therapy would appear to be the mature cells that line the pulmonary airways. These are not rapidly dividing cells; rather, most of them are nonproliferating and many may be terminally differentiated (65). The identification of the progenitor cells in the airway is uncertain. Although CFTR may also be present in submucosal glands (63,64), there is no data as to its function at that site; furthermore, such glands appear to be relatively inaccessible.

Because we are unaware of any method of removing and reimplanting airway epithelial cells that is likely to be possible in humans, a therapeutic strategy based on removal of the target cell from the patient, treatment by gene therapy *in vitro*, and then reimplantation into the patient, seems impractical. Instead, gene therapy for CF appears to require *in vivo* treatment of the airway epithelia.

### 3.2 Delivery of CFTR cDNA

The airway epithelium provides two main advantages for gene therapy. First, access to the airway epithelium can be relatively noninvasive. This is a significant advantage in the development of delivery strategies and it will allow investigators to monitor the therapeutic response. Second, the epithelium forms a barrier between the airway lumen and the interstitium. Thus, application of the vector to the lumen will allow access to the target cell yet, at least to some extent, limit movement through the epithelial barrier to the interstitium and from there to the rest of the body.

### 3.3 Efficiency of Gene Delivery Required to Correct the Genetic Defect

It is unlikely that any gene therapy protocol will correct 100% of the cells that normally express CFTR. However, several observations suggest that correction of a small percent of the involved cells or expression of a fraction of the normal amount of CFTR may be of therapeutic benefit.

- a. CF is an autosomal recessive disease and heterozygotes have no lung disease (1). Thus, 50% of wild-type CFTR would appear sufficient for normal function.
- b. This issue was tested in mixing experiments using CF cells and recombinant CF cells expressing wild-type CFTR (66). The data obtained showed that when an epithelium is reconstituted with as few as 6-10% of corrected cells, chloride secretion is comparable to that observed with an epithelium containing 100% corrected cells. Although CFTR expression in the recombinant cells is probably higher than in normal cells, this result suggests that *in vivo* correction of all CF airway cells may not be required.
- c. Recent observations show that CFTR containing some CF-associated mutations retains residual chloride channel activity (67,68). These mutations are associated with mild lung disease. Thus, even a very low level of CFTR activity may at least partly ameliorate the electrolyte transport abnormalities.
- d. As indicated in our experiments described below (8.2.b.(3).1), complementation of CF epithelia, under conditions that probably would not cause expression of CFTR in every cell, restored cAMP-stimulated chloride secretion.
- e. Levels of CFTR in normal human airway epithelia are very low and are barely detectable. We have not been able to detect it by routine biochemical techniques such as immunoprecipitation or immunoblotting. It is also exceedingly difficult to detect with immunocytochemical techniques (32). Although we have been able to detect CFTR using laser-scanning confocal microscopy in some cases, the signal is at the limits of detection and cannot be detected above background in every case. Despite the minimal levels of CFTR, this small amount is sufficient to generate substantial cAMP-stimulated chloride secretion. The reason that a very small number of CFTR chloride channels can support a large chloride secretory rate is that a large number of ions can pass through a single channel ( $10^6 - 10^7$  ions/sec) (69).
- f. Previous studies using quantitative PCR have reported that the airway epithelial cells contain at most one to two transcripts per cell (70).

### 3.4 Effect of Overexpressing CFTR

Fortunately, gene therapy for CF would appear to have a wide therapeutic index. Just as partial expression may be of therapeutic value, overexpression of wild-type CFTR appears unlikely to cause significant problems. This conclusion is based on both theoretical considerations and experimental results.

Because CFTR is a regulated channel, and because it has a specific function in epithelia, it is unlikely that overexpression of CFTR will lead to uncontrolled chloride secretion. First, secretion would require activation of CFTR by cAMP-dependent phosphorylation. Activation of this kinase is a highly regulated process. Second, even if CFTR chloride channels open in the apical membrane, secretion will not ensue without regulation of the basolateral membrane transporters that are required for chloride to enter the cell from the interstitial space. At the basolateral membrane, the sodium-potassium-chloride cotransporter and potassium channels serve as important regulators of transepithelial secretion (62).

We have expressed human CFTR in transgenic mice under the control of the surfactant protein C (SPC) gene promoter and of the casein promoter (71,72). In those mice, CFTR was overexpressed in bronchiolar and alveolar epithelial cells and in the mammary glands, respectively. Yet despite the massive overexpression in the transgenic animals, there were no observable morphologic or functional abnormalities. (It is known that human CFTR expressed in recombinant mouse cells is functionally active, ref. 35.) In addition, expression of CFTR in the lungs of cotton rats produced no reported abnormalities (11).

### 3.5 Use of Retroviruses for CF Gene Therapy

Most gene therapy protocols that have been presented to the RAC have used recombinant retroviruses. Because of the previous scientific and regulatory experience with retroviruses, it seems appropriate to consider their use for gene therapy of CF.

It is clear that retroviruses can be used to express CFTR in cultured epithelial cells; we have done so (35) as have others (23). However at the present time, several considerations would seem to preclude the use of retroviruses as a vector to treat CF. a) Most previous uses of retrovirus in gene therapy have treated cells *ex vivo*, but removal of target cells seems impractical for treatment of CF lung disease. b) Retroviruses require dividing cells, whereas airway epithelial cells divide infrequently. c) The airway epithelium is likely to contain  $10^{10}$ -  $10^{11}$  (73) epithelial cells. Even if it were possible to produce and deliver sufficient retrovirus vector to correct 1% of these cells *in vivo*; a significant risk of insertional mutagenesis might be incurred.

Thus, retroviruses do not currently appear to be the agent of choice for CF gene therapy.

## 4.0 USE OF ADENOVIRUS FOR CF GENE THERAPY

For our study, we propose to use a recombinant adenovirus as the vector to express CFTR.

### 4.1 Differences Between Adenovirus and Retrovirus Vectors for Gene Therapy

There are many differences between retroviruses (reviewed in 74), which have been used for most gene therapy protocols, and adenoviruses (reviewed in 75-77), the vector proposed here. Adenovirus contains a double stranded DNA genome of approximately 36 kb within a small icosahedral protein virion. The retrovirus genome is a single stranded RNA of approximately 7 kb, within a core surrounded by a lipid-containing envelope. Retroviruses infect cells by converting their genome into double-stranded DNA and integrating into the host cell genome; adenoviruses replicate extrachromosomally. Retrovirus gene expression is chronic and virus shedding continues over the life of the cell. Adenovirus infections lyse permissive host cells but may establish latent infections in others, e.g., lymphocytes and monocytes (78,79). Retroviruses contain minimally 3 genes encoding reverse transcriptase, gag proteins which comprise the protein core, and the envelope protein. Adenoviruses encode a great many more proteins including early proteins which are expressed prior to viral DNA synthesis and late proteins comprising predominantly virion structural proteins. Adenoviruses are stable to purification whereas retroviruses are not.

Because the genome of the retroviruses is so simple, and because the viral gene products can be expressed without deleterious effect on the host cell, it is relatively straight forward to remove the endogenous retrovirus genes and to replace them with marker genes, antibiotic resistance genes or genes encoding therapeutic proteins (10,80). Such vectors can be grown in packaging cells that provide the missing gene functions to produce the

defective retrovirus, albeit at low titer. The adenovirus genome is much more complex and present vectors retain virtually all of the viral genes. The introduced genes replace either a) dispensable viral gene functions (such as early region 3) to produce a live recombinant viral vector, or b) gene functions that can be provided in trans. In the latter case, a transformed human cell line called 293 that expresses the early region 1 genes is available and this can be used to grow the otherwise defective E1-deleted adenovirus vectors (81).

The ends of the retrovirus genome contain so called Long Terminal Repeats (LTR). The LTRs include elements that promote integration and activation of adjacent genes (enhancers). Integration of retrovirus DNA introduces two copies of the LTR. The LTRs activate viral gene expression, but they can also activate cellular genes adjacent to the integration site. This is the basis for insertional mutagenesis involving gene activation by retroviruses (82). Insertional mutagenesis can also occur by gene disruption. The adenovirus genome contains an inverted terminal repeat (ITR) involved in DNA replication, but the viral promoter enhancer elements map outside the repeated sequences. Although integration of adenovirus DNA is not required for virus replication, it can occur at low frequency in non-permissive cells or when the virus is disabled (83-86). However, integration does not obligatorily occur at the terminal repeats. Mutagenesis by gene activation therefore appears less likely, although mutagenesis by gene disruption as with retroviruses remains a possibility. However, we are unaware of any reports of insertional mutagenesis associated with adenovirus integration. Finally, because retrovirus vectors integrate their nucleic acid, gene expression from such a vector potentially persists for the life time of the treated cell. For adenoviruses, the DNA is predominantly extrachromosomal and continued expression may require multiple administrations.

## 4.2 Advantages of Adenovirus as a Vector System

Adenoviruses are widespread in the human population and almost 50 different serotypes are known. Viral infection, especially with the Ad2 and Ad5 serotypes of subgenus C, is not generally associated with serious disease (75,76); infection usually results in mild respiratory tract symptoms. Exposure to the virus is widespread with the majority of adults being seropositive for type C viruses. Engineered adenovirus offer an attractive means by which gene therapy for CF might be achieved (10,11,80).

- a) The virus can be readily manipulated to encode and express the desired gene product, CFTR.
- b) Deletion of viral genes can impair the activity of adenovirus in terms of its ability to replicate in a normal lytic viral life cycle.
- c) Adenovirus has a natural tropism for the target cells of the airway epithelium.
- d) The viruses are able to infect quiescent cells, as are found in the airways.
- e) Adenovirus expression can be achieved without integration of the viral DNA into the host cell chromosome, thereby reducing concerns about insertional mutagenesis.
- f) *In vivo* delivery of  $\alpha 1$  antitrypsin and CFTR using adenovirus vectors has been reported in experiments using cotton rats (11,87).
- g) The molecular biology of human adenoviruses are relatively well understood.
- h) Recombinant adenovirus can be grown to high titer and the virus purified.

### 4.3. Design of the Present Generation Vector

The vector to be utilized in the present protocol is an E1-deficient Ad2 (Fig. 1). It is named Ad2/CFTR-1 and includes the coding sequence for CFTR in place of E1. Removal of E1

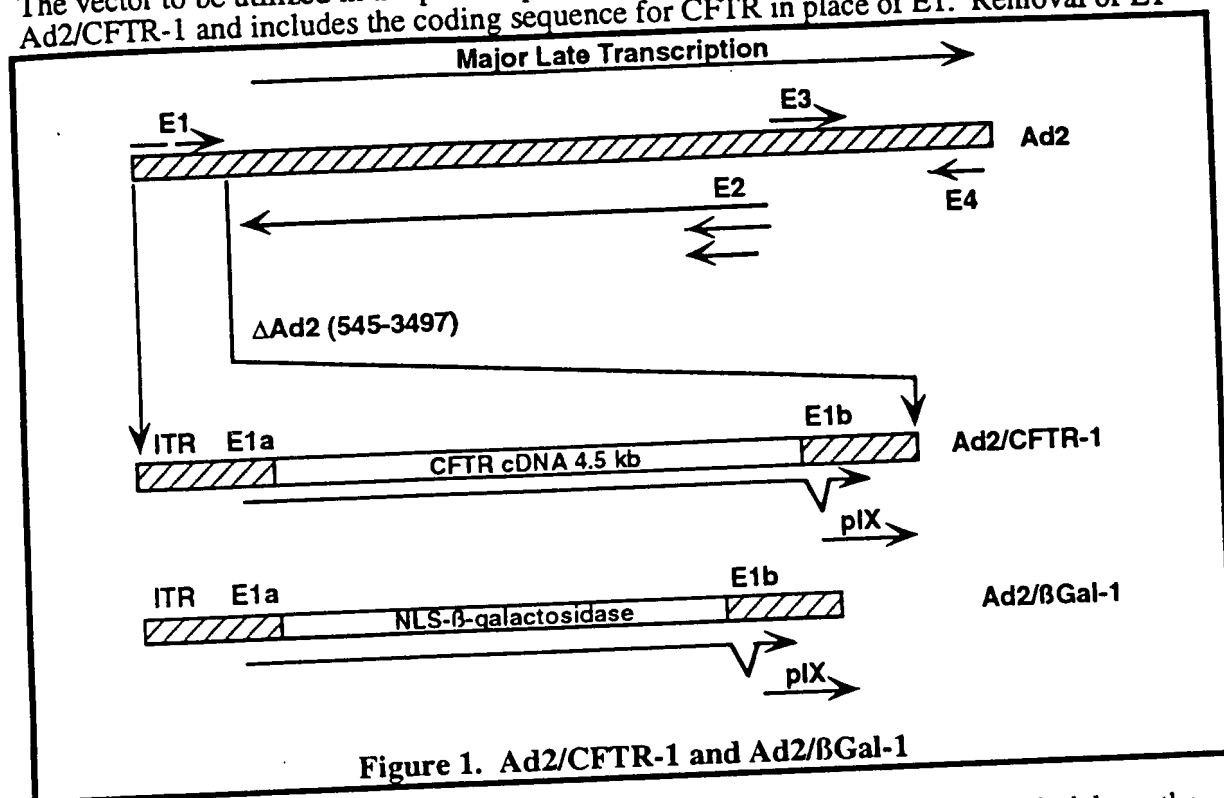


Figure 1. Ad2/CFTR-1 and Ad2/βGal-1

has several advantages: a) it impairs lytic viral replication in human cells; b) it deletes the region of the genome associated with the *in vitro* transforming activity of the virus; and c) it creates space in the genome for the insertion of exogenous DNA. We also constructed a related vector encoding β-galactosidase (Ad2/βGal-1).

Unlike many E1 deleted recombinant adenovirus vectors (10,11,80), Ad2/CFTR-1 retains the E3 region. This region is dispensable, at least for growth in tissue culture, and it is often deleted to allow space for the newly introduced DNA sequences. Since this was not done with Ad2/CFTR-1, its DNA is approximately 104.5% of the length of the wild-type adenovirus DNA. This means that the viral DNA is at the upper limit of size able to package into virions (88,89). For this reason, the Ad2/CFTR-1 virus grows less readily than normal, typically producing 5-10% of the yield obtained in 293 cells with vectors of wild-type size.

The E3 region of the Ad2/CFTR-1 encodes a variety of proteins. One of these proteins, gp19, is believed to interact with and prevent presentation of class I proteins of the major histocompatibility complex (MHC) (reviewed in 90). This property prevents recognition of the infected cells and thus may allow viral latency. The presence of E3 sequences, therefore, has two useful attributes; first, the large size of the viral DNA renders it doubly defective for replication (i.e., it lacks early functions and is packaged poorly) and second, the absence of MHC presentation could be useful in later applications of Ad2/CFTR-1 in gene therapy involving multiple administrations, because it may avoid an immune response to recombinant virus-containing cells.

Not only are there advantages associated with the presence of E3; there may be disadvantages associated with its absence. Studies of E3-deleted virus in animals have suggested that they result in a more severe pathology (91). Furthermore, E3-deleted virus, such as might be obtained by recombination of an E1 plus E3-deleted virus with wild-type virus, is reported to outgrow wild-type in tissue culture (92). In contrast, however, a recent report of an E3 replacement vector encoding hepatitis B surface antigen, suggests that when delivered as a live enteric vaccine, such a virus replicates poorly in humans compared to wild-type (93).

#### 4.4 Safety of Adenovirus as a Gene Delivery System

Adenoviruses have an impressive safety profile in clinical use. Serotypes 4 and 7 have been used as live enteric vaccines for many years with an excellent safety record; they have been used to treat military recruits to prevent epidemic acute respiratory disease (94,95). However, adenoviruses are regarded as tumor viruses in that injection of some strains into newborn rodents can cause tumors and infection of some nonpermissive cells can cause transformation of cellular growth (77). Of the different adenovirus subgenera, subgroup A, including Ad12, is highly oncogenic in newborn animals; subgenus B including Ad7 is weakly oncogenic; and the other subgenera, including Ad2 and Ad5 of subgroup C, are considered non-oncogenic in that they have not been shown to induce tumors upon injection into animals (96,97), reviewed in 75,80). The region of the adenovirus genome associated with oncogenicity is the E1 region, encoding E1a with immortalizing activity and E1b with transforming ability.

The following arguments lead us to believe that the oncogenic potential of adenovirus 2 possesses little or no risk to human patients in the protocol proposed here:

- a. Ad2 belongs to subgenus C that is regarded as non-oncogenic.
- b. The genes with transforming potential have been deleted from our vector.
- c. Human use of the mildly oncogenic subgenus B Ad7 strain as a live, enteric, human vaccine has proved safe in millions of young adults over many years (94,95).
- d. Even the so called highly oncogenic strains of virus are not particularly efficient at causing tumors in rodents, requiring many millions of virus particles per event (80).
- e. No naturally occurring tumors in any animal have been shown to be caused by adenoviruses even though over 100 different avian and mammalian serotypes are known (80).
- f. An extensive survey of human tumors revealed no evidence of adenovirus DNA associated with neoplasia (75,98,99).

#### 4.5 Potential Disadvantages of Adenovirus for CF Gene Therapy

Adenovirus has some potential disadvantages for gene therapy.

- a. Administration of the virus may induce an immune response. An immune response could reduce the efficacy of the virus by producing neutralizing antibodies.
- b. Of more immediate concern, an immune response could stimulate an inflammatory response to the virus which could exacerbate problems in lung function in patients who already have compromised activity.

c. Another potential problem with the administration of a live, though disabled virus, is the possibility of virus replication. If replication were to occur in the patient, virus could spread to other persons and to the environment. Replication in the patient could occur, by a variety of means. The adenovirus vector itself, though disabled, may have some low level ability to replicate. The missing E1 functions could be provided by coinfection with another adenovirus; by coinfection with another virus able to provide E1-like functions; by expression of E1 resulting from an earlier perhaps now latent infection (100); or by host cell proteins, which though not directly related to the E1-encoded proteins, nevertheless were able to mimic their activity (101,102). In addition to complementation of viral activity, a further possibility is that the defective virus could recombine with a related virus either by homologous recombination or by illegitimate means so as to create a new viral entity with novel biological properties.

d. The adenovirus vector will require repeated administration, since we do not expect to target a progenitor cell and we expect vector expression to decrease with time. Such repeated administration will likely be necessary for almost all approaches to gene therapy for CF.

In Section 8.0 (the NIH Points to Consider), we address these issues of inflammation and replication and describe animal and tissue culture safety experiments. Those results lead us to believe that the potential risk posed by these factors is small and that our protocol both minimizes such risks and allows their assessment.

## **5.0 USE OF NASAL EPITHELIUM FOR STUDIES OF GENE THERAPY**

For our first studies of gene therapy, we propose to use the nasal epithelium for several important reasons.

### **5.1 Nasal and Pulmonary Epithelia Have Similar Morphology and Function.**

As indicated above, disease of the pulmonary airways is the major cause of morbidity and mortality in CF. However, the upper airway epithelium that lines the nasal cavity is similar in morphology and function to the airway epithelium that lines the lower pulmonary airways.

Most importantly for our studies, CF causes similar abnormalities of electrolyte transport in both. Both manifest the lack of CFTR chloride channel function. In fact, the first evidence to suggest that CF airway epithelia have defective electrolyte transport, was a study of nasal airway epithelium (103). Since then, many *in vitro* and *in vivo* studies have used the nasal epithelium as a model of the lower respiratory tract epithelium.

### **5.2 Use of Nasal Epithelium Provides Advantages for Safety.**

a. Use of the nasal epithelium will allow us to use a very small total amount of virus because we can apply it to a limited area. As a result, we will be able to obtain a great deal of useful information, but at the same time, use of a small amount of virus will minimize the risk.

b. Use of the nasal epithelium for our first studies of the recombinant virus has the advantage that the epithelium is more readily accessible than that in the lower airway for

the frequent studies required to test safety and efficacy. Because it is more accessible, there will be less patient discomfort and risk.

c. Use of the nasal epithelium will allow us to obtain a biopsy of the precise area to which the recombinant virus was applied. This will allow an assessment of cytopathic effects and the inflammatory response.

d. If significant inflammation or cytotoxicity should develop as a result of administration of the recombinant virus, the consequences to the patient will be much less severe if they occur in the nasal epithelium than if they occur more generally in the lung.

### 5.3 Use of Nasal Epithelium Provides Advantages for Assessing Efficacy.

Measurement of the transepithelial electrical potential difference across the nasal epithelium provides an easy and reliable measure of the chloride transport function of the epithelium (103,104). The test is noninvasive; it only involves the use of a small polyethylene tube to probe the voltage generated across the nasal mucosa. Moreover, the test can be repeated frequently and there is little discomfort to the patient. Use of the nasal epithelium will also allow us to assess the presence of mRNA and protein. Thus, the functional and biochemical evaluation of recombinant virus application can be more readily performed with less risk to the patient in the nasal epithelium than in the pulmonary epithelium.

## 6.0 IS THE USE OF AN ADENOVIRUS VECTOR SAFE?

Although live adenovirus has been used for vaccine purposes and an E3 replacement recombinant adenovirus has been tested in limited clinical trials (93), there is clearly concern as to whether the use of an E1 replacement vector will be safe. These issues are dealt with in detail in Section 8.0, the NIH Points to Consider. Here we highlight what we believe to be novel aspects of using adenovirus in human gene therapy.

### 6.1 Will the Virus Cause Immediate Damage?

Replication of wild-type Ad2 is usually associated with only minor respiratory ailments; the vector to be used here is disabled for replication. Doses in excess of  $10^4$  times the proposed human dose (on a mass/kg basis) have been tested in monkeys and hamsters with only a minor transient inflammatory response.

### 6.2 Will the Virus Cause Tumors?

Although some adenoviruses can cause tumors in newborn animals, no naturally occurring tumor in animals or humans has ever been associated with any adenovirus strain (80,98,99). The adenovirus genes associated with cellular transformation in tissue culture are deleted. Although adenovirus DNA can integrate, this is not part of the normal viral replication cycle and there is no evidence for insertional mutagenesis.

### 6.3 Will the Virus Replicate?

The vector proposed here is doubly defective: it lacks a crucial early viral gene, E1, required for viral replication and it contains a genome of 104.5% normal size which renders it difficult to package. Although we present data for limited viral DNA synthesis, we have no evidence for replication of the adenovirus vector in tissue culture cells, in monkeys, or

in hamsters at doses that in some cases greatly exceed the proposed human dose, both in terms of total virus added and multiplicity of infection per cell.

#### **6.4 Will the Virus Recombine?**

Homologous recombination occurs at reasonable frequency within adenoviruses of the same subgroup (105), but the products of such recombination would be very similar to the starting materials. Recombination between subgroups does not appear to occur. The only viable products of non-homologous or illegitimate recombination would necessarily contain E1-like sequences and delete other sequences in viral or CFTR coding regions. Such products would likely be overgrown by wild-type virus and subsequently become self limiting. To ensure this, we will only treat patients seropositive for Ad2.

#### **6.5 Will Virus Growth Be Complemented?**

The growth of the E1-deleted vector could be complemented by provision of E1 gene function. Growth of the present vector is so disabled, however, that even under the most favorable conditions, wild-type virus rapidly overgrows the defective virus. Complementation by provision of a cellular protein with E1-like activity is a theoretical possibility (101,102). We have not detected virus replication in tissue culture cells or in animals, but this possibility cannot be discounted. For this reason we plan as an exclusion criterion the presence of E1 sequences detectable by PCR in the nasal brush samples taken prior to treatment.

#### **6.6 Will the Virus Be Sufficiently Pure?**

To alleviate the possibility of introducing another human virus along with the adenovirus, the parent cell lines and the viral seed stock will be extensively tested for biological agents. We will also test for cellular transforming activity in the preparations. A PCR procedure capable of detecting 1 part wild-type virus in  $10^8$  E1 deleted virus will also be used. The presence of wild-type virus below this level perhaps originating from recombination between Ad2/CFTR-1 and the endogenous Ad5 E1 sequences in 293 cells should become apparent in the extended *in vitro* viral tests on the virus seed stock.

#### **6.7 Will Administration Be Immunogenic?**

Our data in monkeys and hamsters show that they mount an immune response to introduced adenovirus vector. This could lead to a reduction of efficacy in protocols requiring multiple administrations and we plan to test this in animals. However, for the present human protocol we plan only a single application in each patient.

#### **6.8 Does the Protocol Minimize Risk?**

The present protocol proposes to treat 3 patients and to use minimal amounts of virus consistent with the gathering of safety and limited efficacy data. We believe that the provisions for the health of the patients, their care givers, visitors, and the public do minimize risk.

## 7.0 SPECIFIC AIMS

The foregoing considerations indicate that gene therapy would be a major advance in the treatment of CF. They also suggest that adenovirus may be a good vector system for correcting the CF pulmonary defect. In section 8.0 (the NIH Points to Consider), we provide data that a recombinant adenovirus encoding CFTR can be used to complement the CF airway epithelial electrolyte transport abnormality. We also provide safety data for the vector.

Thus, we believe that it is now appropriate to test the feasibility of gene therapy in CF patients. We believe an appropriately designed protocol which uses minimal amounts of adenovirus and which is designed to minimize the risk to the patients is of critical importance in directing future research. Other approaches might be taken to introduce DNA into the airway cells of CF patients if adenovirus proves ineffective. On the other hand, there are many future generations of adenovirus that can be envisaged that would improve its properties as a gene delivery vector. Thus, there is an urgent need for data in humans to guide which direction future research should take. We believe the present protocol would provide such guidance, but at the same time, minimize any possible risk to the patient.

There are three specific aims of the proposed study.

- 7.1 Assess safety of the current recombinant adenoviral vector/CFTR gene construct when applied to human airway epithelium *in vivo*. This aim will assess the possibility that the recombinant virus will produce local inflammation or injury of the airway epithelium. It will also determine the time after administration that the live virus disappears from the nasal epithelium and will test for any possible subsequent virus replication.
- 7.2 Assess efficacy of the recombinant adenoviral vector/CFTR gene construct in human airway epithelium *in vivo*. This aim will assess the ability of the viral construct to deliver the cDNA for CFTR to respiratory epithelium *in vivo*, the ability of the transferred cDNA to direct the expression of CFTR protein, the ability of that process to correct the CF chloride transport defect, and the duration of these effects.
- 7.3. Assess the effect of dose of recombinant adenoviral vector/CFTR gene construct on safety and efficacy.

## 8.0 NIH POINTS TO CONSIDER

### I. DESCRIPTION OF PROPOSAL

#### A. OBJECTIVES AND RATIONALE OF THE PROPOSED RESEARCH.

State concisely the overall objectives and rationale of the proposed study. Please provide information on the specific points that relate to whichever type of research is being proposed.

The overall objectives of the proposed study are to use a recombinant adenovirus to deliver CFTR to the nasal epithelium of CF patients to: a) assess the safety of this adenovirus vector when applied to human airway epithelium *in vivo*; b) assess the efficacy in correcting the chloride transport defect *in vivo*; and c) assess the effect of dose of recombinant adenovirus on both safety and efficacy. The results will also provide guidance for the future direction of CF gene therapy.

The rationale for the proposed study is as follows: CF is the most common lethal genetic disease of Caucasians (1-3). Despite current standard therapy of CF, the median age of survival is only 26 years. CF is caused by mutations in the gene encoding CFTR (4-6). Recently obtained knowledge of the gene that encodes CFTR, an understanding of the function and biochemistry of the protein product (7), and insight into the molecular basis of the disease (32,48,57-59), suggests that gene replacement therapy could represent an important advance in the treatment of this disease. Eventually, we hope to use recombinant adenovirus to deliver CFTR to the airway epithelium. In our initial studies, we will target the nasal epithelium, because it will allow us to test both efficacy and safety with the least risk to the patients.

##### A.1. Use of Recombinant DNA for Therapeutic Purposes.

Our present protocol will not be of therapeutic benefit to the participants. Correction of the genetic defect is likely to be limited to the area of virus application in the nose and to be transient. However, we believe the information obtained from this study regarding safety, efficacy, and dosing are critical before studies of bronchial administration can be safely performed. They will also guide the design of future generations of adenovirus.

##### A.1.a. Why is the disease selected for treatment by means of gene therapy?

Despite current standard therapy, CF remains a lethal disease. There is no treatment that corrects the basic defect. Nevertheless, the location of the target tissue, the understanding of the biology of CFTR, and the vector/delivery system provide an excellent opportunity for safe gene replacement therapy.

##### A.1.b. Describe the natural history and range of expression of the disease selected for treatment. What objective and/or quantitative measures of disease activity are available? In your view, are the usual effects of the disease predictable enough to allow for meaningful assessment of the results of gene therapy?

We describe the natural history in Section 2.1. Disease activity is routinely measured with history and physical examination, chest X-rays, pulmonary function tests, and sputum cultures. The basic defect in the airway epithelium can be assessed biochemically and functionally by measurement of the transepithelial electrical potential difference across the nasal epithelium (103,104). The biochemical defects to be measured here are very

predictable. Furthermore, clinical studies suggest that the clinical course is sufficiently predictable to allow for the meaningful assessment of future protocols aimed at delivering the gene to airway epithelia.

**A.1.c. Is the protocol designed to prevent all manifestations of the disease, to halt the progression of the disease after symptoms have begun to appear, or to reverse manifestation of the disease in seriously ill victims?**

The protocol is designed to test the safety, efficacy and dose of Ad2/CFTR-1. Because application will be limited to the nasal epithelium, it will not reverse manifestations of disease.

**A.1.d What alternative therapies exist? In what groups of patients are these therapies effective? What are their relative advantages and disadvantages as compared with the proposed gene therapy?**

We described standard therapies for CF in Section 2.2. At present there are no therapies designed to correct the fundamental defect. Participants will continue with all their current therapies during the course of the study.

**A.2. Transfer of DNA for Other Purposes.**

Not applicable.

**B. RESEARCH DESIGN, ANTICIPATED RISKS AND BENEFITS.**

**B.1. Structure and characteristics of the biological system.**

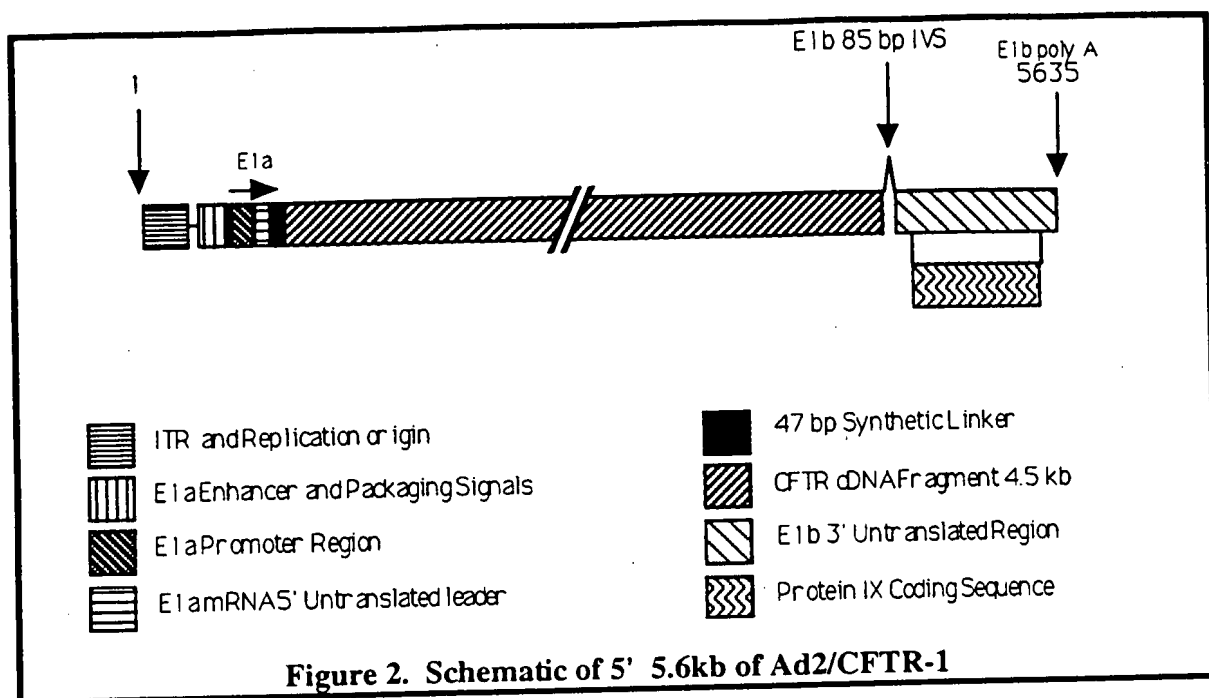
Provide a full description of the methods and reagents to be employed for gene delivery and the rationale for their use. The following are specific points to be addressed:

**B.1.a. What is the structure of the cloned DNA that will be used?**

**B.1.a.(1) Describe the gene (genomic or cDNA), the bacterial plasmid or phage vector, and the delivery vector (if any). Provide complete nucleotide sequence analysis or a detailed restriction enzyme map of the total construct.**

The cloned DNA to be used in the protocol was derived from Ad2 DNA and fragments of CFTR DNA. All of the DNA samples were originally obtained from ATCC and the construct assembled at Genzyme. The DNA construct comprises a full length copy of the Ad2 genome of approximately 37.5 kb, from which the early region 1 genes (present at the 5' end of the viral DNA) have been deleted and replaced by the cDNA for CFTR. Specifically, nucleotides 546 to 3497 of Ad2 DNA are replaced with nucleotides 123-4622 of the published CFTR sequence with 53 additional linker nucleotides. The topology of the 5' end of the recombinant molecule is illustrated in Figure 2. The nucleotide sequence of the portion of Ad2 molecule that has been manipulated is given as Appendix 8. The remainder of the viral DNA is published in reference 106.

The predicted CFTR transcript comprises a hybrid 5' untranslated region containing 46 nucleotides of Ela upstream sequences, 47 bp of sequences derived from synthetic linkers and 10 nucleotides derived from the CFTR insert. The CFTR coding sequence comprises nucleotides, 603-5045 of the recombinant virus and 104-4546 of the hybrid Ela-CFTR-Elb



mRNA. Within the CFTR cDNA there are two differences from the published (5) cDNA sequence. An A to C change at position 1990 of the CFTR cDNA (published CFTR cDNA coordinates) which was an error in the original published sequence, and a T to C change introduced at position 936. The change at position 936 is silent but increases the stability of the cDNA when propagated in bacterial plasmids (22,57). The 3' untranslated region comprises 6 bp of linker sequences and approximately 485 nucleotides (following removal of spliced out nucleotides) derived from the E1b mRNA. Although the adenovirus protein IX coding sequence is embedded within the 3' E1b derived untranslated region, the protein IX transcript is independent of the CFTR transcript.

Although the activity of CFTR can be measured readily by electrophysiological methods, it is relatively difficult to detect biochemically or immunocytochemically, particularly at low levels of expression (22,32). We, therefore, constructed a very similar adenovirus vector encoding  $\beta$ -galactosidase containing a nuclear localization signal. This vector is useful for some efficacy and marking experiments, and also for safety studies. The Ad2/ $\beta$ Gal-1 vector is identical to the CFTR virus, except that in place of the CFTR cDNA, an approximately 3.3 kb sequence encoding *E. coli*  $\beta$ -galactosidase fused to an SV40 T-antigen nuclear localization signal was inserted (107).

Because this vector is smaller (approximately 101% of wild-type) than Ad2/CFTR-1, it replicates more readily in 293 cells (i.e.,  $10^8 - 10^9$  PFU/ml vs.  $10^7 - 10^8$  PFU/ml for Ad2/CFTR-1). For some safety studies, we used the Ad2/ $\beta$ Gal-1 virus as a more sensitive means for testing the possibility of vector replication.

**B.1.a.(2) What regulatory elements does the construct contain (e.g., promoters, enhancers, polyadenylation sites, replication origins, etc.)? From what source are these elements derived? Summarize what is currently known about the regulatory character of each element.**

All the regulatory elements used in the construct are the endogenous Ad2 control elements. The promoter/enhancer responsible for transcription of the CFTR cDNA is the E1a

promoter. This is known to be expressed at moderate levels in a wide variety of cells. Termination/polyadenylation occurs at the site normally used by the E1b and protein IX transcripts. An endogenous 84 bp intron is present between the coding sequences for CFTR and protein IX.

The regulation of the E1a promoter has been studied extensively, since it was one of the earliest and most readily available eukaryotic transcriptional control regions (108-110, reviewed in 111). The transcription factors that bind the E1a promoter are well characterized (reviewed in 112,113). The promoter has been transferred into a wide variety of cells and shown to be active in transcription of marker genes. Promiscuous expression perhaps reflects the role of E1a in the early stages of viral replication where it might encounter different host cells in various stages of proliferation (77).

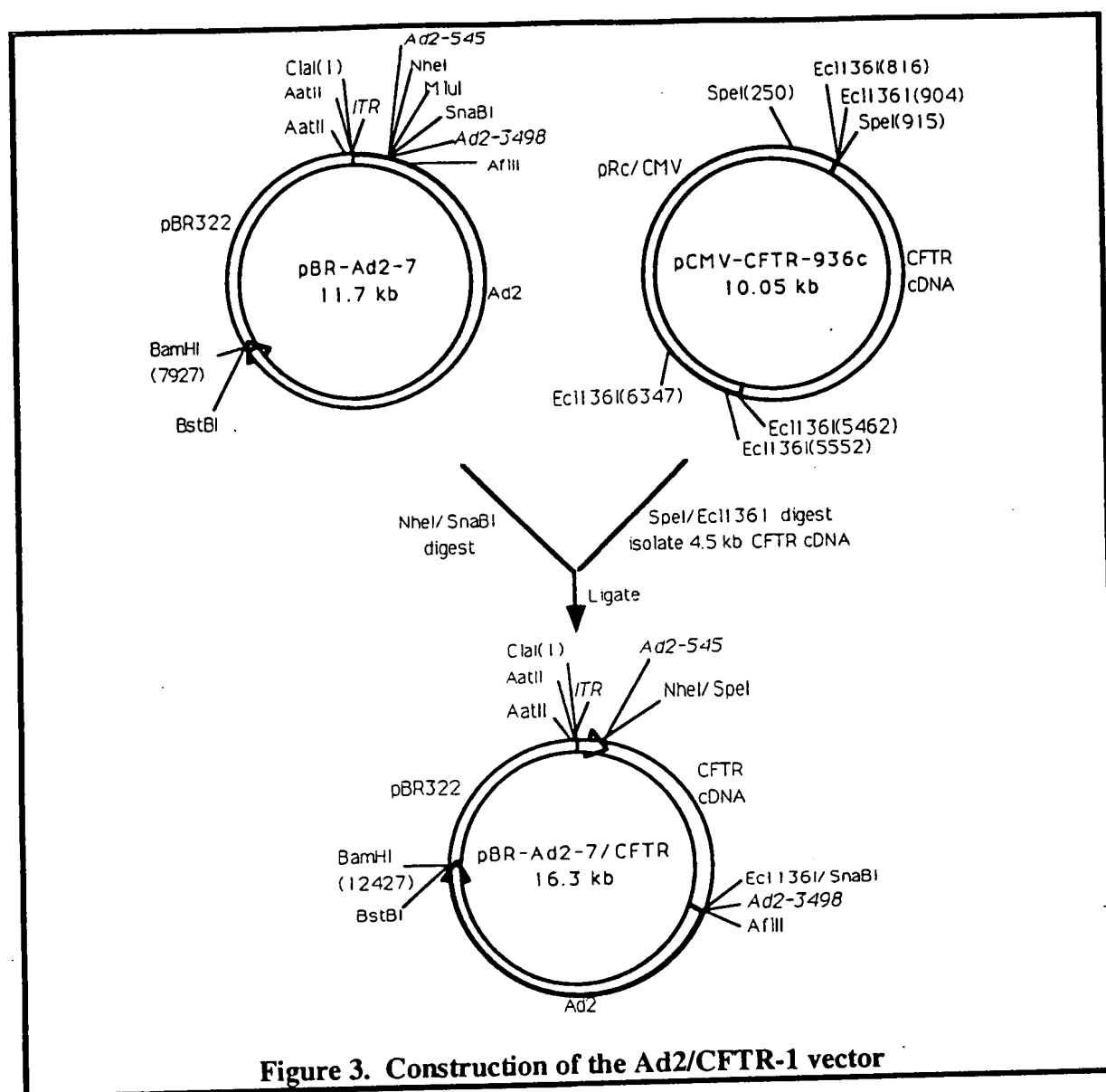
Adenovirus DNA replication has been extensively studied (reviewed in 75 and in 114) and *in vitro* systems for replication of adenovirus DNA have been developed (115,116). These studies have allowed the dissection of the adenoviral DNA replication process and have identified virally encoded proteins of the E2 transcriptional unit involved in DNA replication as well as a number of host cell encoded factors involved in the process. In addition, the viral DNA sequences within the terminal repeats which are required for DNA replication have been defined for Ad2, Ad5, and Ad4 (117,118). The relative permissivity of a specific cell to adenovirus DNA replication is thus a result of complex interactions between host-encoded nuclear factors, virally encoded replication machinery and specific sequences within the terminal repeats.

In addition, in quiescent cells, replication of viral DNA is dependent upon the ability of the virus to stimulate the infected cell into a proliferative state able to support efficient viral replication (77). It has been proposed that one of the functions of the adenovirus E1a-12S protein is to induce infected cells to enter such a proliferative state by displacing transcription factor E2F from the retinoblastoma gene product, thereby leading to activation of genes required for efficient DNA synthesis as well as viral transactivation (119). E4 gene products also play a role in activation of E2F (120). Thus, as for DNA synthesis, adenovirus gene expression is a complex series of cooperative reactions involving both host cell proteins and multiple viral gene products. Cells that are already in a proliferative state may be capable of supporting limited adenovirus replication in the absence of E1a transactivation. Therefore, experiments with proliferating cells in tissue culture may not accurately reflect the replicative ability of an E1a-deleted virus upon infection of a quiescent cell.

In addition to the elements expected to be involved in the expression and replication of the Ad2/CFTR-1 vector, many other regulatory sequences are present within the remainder of the Ad2 genome. Expression of these sequences is expected to be low and to have little influence on CFTR expression.

### **B.1.a.(3) Describe the steps used to derive the DNA construct.**

Construction of the recombinant Ad2/CFTR-1 virus was accomplished as follows (Fig. 3 and 4). The CFTR cDNA was excised from the plasmid pCMV-CFTR-936C using restriction enzymes SpeI and Eco136I. pCMV-CFTR-936C consists of a minimal CFTR cDNA encompassing nucleotides 123-4622 of the published CFTR sequence cloned into the multiple cloning site of pRC/CMV (Invitrogen Corp.) using synthetic linkers. The



CFTR cDNA within this plasmid has been completely sequenced. The SpeI/Ecl136I restriction fragment contains 47 bp of 5' sequence derived from synthetic linkers and the multiple cloning site of the vector.

The CFTR cDNA was inserted between the NheI and SnaBI restriction sites of the adenovirus gene transfer vector pBR-Ad2-7. pBR-Ad2-7 is a pBR322 based plasmid containing an approximately 7kb insert derived from the 5' 10680 bp of Ad2 inserted between the Clal and BamHI sites of PBR322. From this Ad2 fragment, we have deleted sequences corresponding to Ad2 nucleotides 546-3497 and replaced them with a 12 bp multiple cloning site containing an NheI site, an MluI site, and a SnaBI site. The construct also contains the 5' inverted terminal repeat and viral packaging signals, the E1a enhancer and promoter, the E1b 3' intron and the 3' untranslated region and polyadenylation sites. The resulting plasmid was called pBR-Ad2-7/CFTR. Its use to assemble virus is described in B1b(1)b.

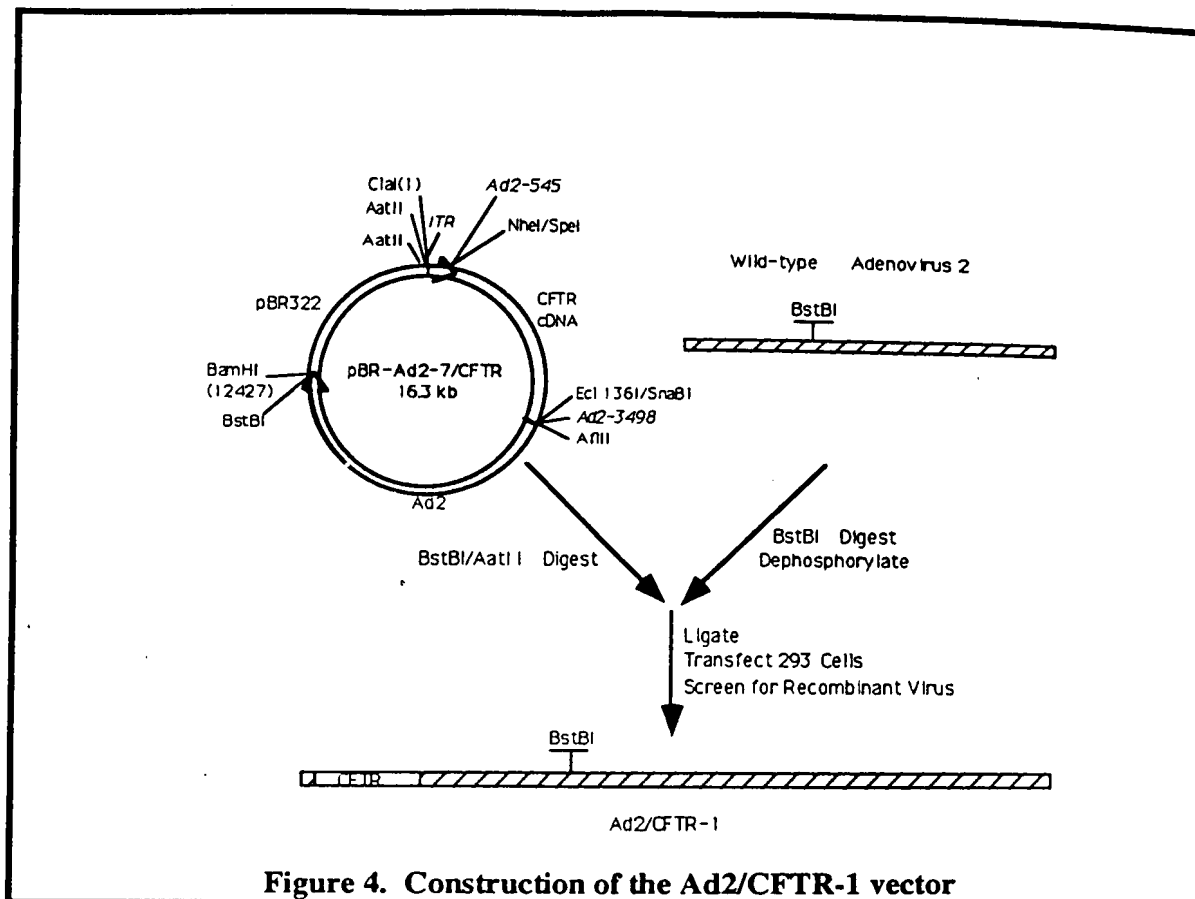


Figure 4. Construction of the Ad2/CFTR-1 vector

B.1.b. What is the structure of the material that will be administered to the patient?

B.1.b.(1) Describe the preparation, structure, and composition of the materials that will be given to the patient or used to treat the patients' cells.

B.1.b.(1)(a) If DNA, what is the purity (both in terms of being a single DNA species and in terms of other contaminants)? What tests have been used and what is the sensitivity of the tests?

Not applicable

B.1.b.(1)(b) If a virus, how is it prepared from the DNA construct? In what cell is the virus grown (any special features)? What medium and serum are used? How is the virus purified? What is its structure and purity? What steps are being taken (and assays used with their sensitivity) to detect and eliminate any contaminating materials (for example, VL30 RNA, other nucleic acids, or proteins) or contaminating viruses (both replication-competent or replication-defective) or other organisms in the cells or serum used for preparation of the virus stock including any contaminants that may have biological effects?

## 1. Virus preparation from DNA

To generate the recombinant Ad2/CFTR-1 adenovirus, the vector pBR-Ad2-7/CFTR was cleaved with BstB1 at the site corresponding to the unique BstB1 site at 10670 in Ad2 (Fig. 4). The cleaved plasmid DNA was ligated to BstB1 restricted Ad2 DNA. Following ligation, the reaction was used to transfect 293 cells by the calcium phosphate procedure. Approximately 7-8 days following transfection, a single plaque appeared and was used to reinfect a dish of 293 cells. Following development of cytopathic effect (CPE), the medium was removed and saved. Total DNA was prepared from the infected cells and analyzed by restriction analysis with multiple enzymes to verify the integrity of the construct. Viral supernatant was then used to infect 293 cells and upon development of CPE, expression of CFTR was assayed by the cAMP-dependent protein kinase (PKA) immunoprecipitation assay (22). Following these verification procedures, the virus was further purified by two rounds of plaque purification.

Plaque purified virus was grown into a small seed stock by inoculation at low multiplicities of infection onto 293 cells grown in monolayers in 925 medium supplemented with 10% bovine calf serum (details below). Material at this stage was designated a Research Viral Seed Stock (RVSS) and was used in all preliminary experiments.

## 2. Virus Host Cell

Ad2/CFTR-1 is propagated in human 293 cells (ATCC CRL1573). These cells are a human embryonal kidney cell line which were immortalized with sheared fragments of human Ad5 DNA. This cell line was established in the early 1970s in Leiden, Holland prior to widespread HIV infection in the population. The 293 cell line expresses adenovirus early region 1 gene products and consequently, supports the growth of E1 deficient adenoviruses. By analogy with retroviruses, 293 cells could be considered a packaging cell line, but they differ from usual retrovirus lines in that they do not provide missing viral structural proteins; rather, they provide only some missing viral early functions.

Production lots of virus are propagated in 293 cells derived from the Working Cell Bank (WCB). The WCB is in turn derived from the Master Cell Bank (MCB) which was grown up from a fresh vial of cells obtained from ATCC. Because 293 cells are of human origin, they are being tested extensively for the presence of biological agents. The MCB and WCB are being characterized for identity and the absence of adventitious agents by Microbiological Associates, Rockville, MD. The tests to be performed on the MCB and WCB are summarized below. Results of these tests, and similar tests, will be reviewed by the FDA before beginning the clinical trial.

## 3. Tests for the 293 Master Cell Bank

Sterility - Tests for the presence of bacterial and fungal contaminants will be performed according to 21 CFR 610.12.

Mycoplasma - Tests for the presence of agar-cultivable and non-cultivable mycoplasma will be performed according to guidelines outlined in the FDA "Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals (1987), Attachment: Recommended Test Procedures for Mycoplasmas".

Identity - The species of origin of the 293 cells will be identified by means of isozyme and cytogenetic analyses.

**Retroviral Particles** - The presence of retroviral particles will be assessed by electron microscopic analysis of fixed, embedded, and sectioned 293 cell pellets.

***In Vitro* Viral Test** - The presence of viral contamination will be evaluated by cultivation of 293 cell lysates on MRC-5 (ATCC CCL171) and VERO (ATCC CCL81) cells. The presence of viral contaminants will be assessed by screening for cytopathic effects (CPE) and hemagglutination/hemadsorption after 14 days of culture. To enhance sensitivity of this assay, blind passage of inoculated cell cultures will also be performed.

***In Vivo* Viral Test** - The presence of inapparent viral contamination will be evaluated using a number of *in vivo* indicator systems, including adult mice, suckling mice, guinea pig, and embryonated hen eggs (yolk sac and allantoic routes of administration). These tests will be made by direct inoculation for all test systems, as well as blind passage into additional suckling mice and embryonated eggs. All animals and eggs will be monitored for morbidity and mortality.

**Bovine Viruses** - The presence of bovine viral contamination will be evaluated by cultivation of 293 cell lysate on BT cells (bovine turbinate, ATCC CLR 1390). This test will detect the presence of bovine viral diarrhea virus, infectious bovine rhinotracheitis, bovine adenovirus, bovine parvovirus, and parainfluenza 3. After 14 days, the cultures will be examined for CPE and screened for the presence of viral antigens with immunofluorescence localization techniques.

**Porcine Parvovirus** - The presence of porcine parvovirus originating from trypsin used to passage cells will be evaluated by the cultivation of 293 cell lysate on PT-1 (porcine testicular) cells. After 14 days, the cultures will be examined for CPE and screened for the presence of viral antigen with immunofluorescence localization techniques.

**Human Epstein - Barr Virus** - The presence of EBV contamination will be evaluated by Southern blot analysis.

**Human cytomegalovirus** - The presence of CMV contamination will be evaluated by the cultivation of 293 cell lysate on MRC-5 cells. Cultures will be examined for CPE and screened for the presence of viral antigen with immunofluorescence localization techniques. Blind passage of inoculated cultures will be performed at 21-28 days.

**Human Hepatitis B** - The presence of Hepatitis B contamination will be determined by testing of 293 cell culture supernatants for Hepatitis B surface antigen with a monoclonal ELISA technique.

**Human Immunodeficiency Virus** - The presence of HIV will be evaluated by the inoculation of 293 cell lysates onto phytohemagglutinin stimulated human peripheral blood lymphocytes. The cultures will be monitored for CPE and syncytium formation. Culture supernatants will be evaluated by ELISA for the presence of HIV-1 p24 antigen.

**Human parvoviruses** - The presence of adeno-associated virus and B19 contamination will be evaluated by Southern Blot analysis.

#### 4. Tests for the 293 Working Cell Bank

The WCB will be tested for sterility and mycoplasma.

## 5. Medium and Serum

The 293 cells are grown in 925 medium. This is a proprietary protein-free medium developed at Genzyme for the growth of recombinant cells and has been used to produce a variety of recombinant proteins, two of which are currently in human clinical trials. The serum used is 10% Donor Calf Serum (DCS) obtained from vendors which utilize only non-European sources. The DCS is certified by the vendor to be free of adventitious viral agents, including bovine diarrhea virus, parainfluenza 3, infectious bovine rhinotracheitis, and other agents capable of causing cytopathic effects. Porcine trypsin used in the cell culture process is also certified by the vendor to be free of porcine parvovirus and mycoplasma.

## 6. Growth of Virus Seed

To generate a Master Viral Seed Stock (MVSS), a sample of an RVSS shown to contain the required recombinant is propagated on a fresh batch of human 293 cells obtained from ATCC at the same time as, and of the same lot as used to initiate the MCB. Details of the conditions for growth of virus are given below.

## 7. Tests for Master Viral Seed Stock (to be performed by Microbiological Associates)

The MVSS will be characterized by Microbiological Associates, Rockville, MD, and Genzyme, Framingham, MA. The test to be performed by Microbiological Associates are listed below. Summaries of these tests are listed above in the Master Cell Bank characterization section.

Sterility

Mycoplasma

Retrovirus-Electron Microscopy

*In Vitro* Virus

Human Viruses

EBV

CMV

HIV

Parvoviruses: AAV, B19

## 8. Tests for Master Viral Seed Stock (to be performed by Genzyme)

Identity - Restriction maps of DNA from the viral seed stock will be compared to restriction fragment sizes predicted by DNA sequence of the viral vector. SDS polyacrylamide gel electrophoresis for identification of major viral proteins will also be performed.

Purity - The absence of wild-type adenovirus will be assessed by PCR analysis of the viral seed stock. For PCR screening three sets of primers are used. The first hybridizes to nucleotides 3076 to 3092 and 4487 to 4482 and produces a 1.4 kd band specific for wild-type Ad2 and Ad5. The primers of this set map in E1b and sequences 3' to early region 1. In consequence, a signal is not obtained from E1 deleted recombinant viruses nor from the E1 region integrated in 293 cells. The second set is specific for E1a and hybridizes to nucleotides 771 to 788 and 1144 to 1129 of Ad2 to produce a 373 bp band. This primer set will also detect Ad5 and the integrated sequences in 293 cells. A third set hybridizes to the viral E4 at nucleotides 33178 to 33196 and 34032 to 34065 to generate an approximately 900 bp band.

Mixing experiments adding wild-type Ad2 sequences to preparations of E1-deleted virus with primer set 2 indicate that we can detect 10 molecules of wild-type DNA in the presence of  $10^9$  particles of E1-deficient virus.

**Concentration** - The total particle concentration of the viral seed stock will be measured by absorbance at 260 nm ( $A_{260}$ ), where  $1.0 \text{ OD } (A_{260}) = 1.1 \times 10^{12}$  particles.

**Activity** - The infectious unit concentration of the viral seed stock will be measured by end-point CPE titration assays and verified using immunofluorescence using FITC-conjugated Ad antibody (Chemicon).

## 9. Growth of Production Lots of Virus

Production lots of Ad2/CFTR-1 will be produced by inoculation of approximately 5 to  $10 \times 10^7$  PFU of MVSS onto approximately  $1-2 \times 10^7$  WCB 293 cells grown in a T175 flask containing 25 mls of 925 medium. Inoculation is achieved by direct addition of the virus (approximately 2-5 mls) to each flask. Batches of 50-60 flasks constitute a lot.

Following 40-48 hours incubation at  $37^\circ\text{C}$ , the cells are shaken loose from the flask and transferred with medium to a 250 ml centrifuge bottle and spun at 1000 xg. The cell pellet is resuspended in 4 ml phosphate buffered saline containing 0.1 g/l  $\text{CaCl}_2$  and 0.1 g/l  $\text{MgCl}_2$ . The protease inhibitors aprotinin (0.1  $\mu\text{g/ml}$ ) and leupeptin (0.5  $\mu\text{g/ml}$ ) are added and the cells subjected to two cycles of freeze-thaw to release virus. Cellular debris is removed by centrifugation at 1000 xg for 15 min. The supernatant from this centrifugation is layered on top of the  $\text{CsCl}$  step gradient: 2 ml 1.4 g/ml  $\text{CsCl}$  and 3 ml 1.25 g/ml  $\text{CsCl}$  in 10 mM Tris, 1 mM EDTA (TE) and spun for 1 hour at 35000 rpm in a Beckman SW41 rotor. Virus is then removed from the interface between the two  $\text{CsCl}$  layers, mixed with 1.35 g/ml  $\text{CsCl}$  in TE and then subjected to a 2.5 hour equilibrium centrifugation at 75,000 rpm in a TLN-100 rotor. Virus is removed by puncturing the side of the tube with a hypodermic needle and gently removing the banded virus. To reduce the  $\text{CsCl}$  concentration, the sample is dialyzed against 2 changes of 2 liters of Tris buffered saline.

Following this procedure, dialyzed virus is stable at  $4^\circ\text{C}$  for a few days or can be stored for longer periods at  $-80^\circ\text{C}$ . Aliquots of material for human use will be tested and while awaiting the results of these tests, the remainder will be stored frozen. The tests to be performed are described below:

## 10. Tests for Virus Productions Lots (to be performed by Genzyme)

**Identity** - Restriction map and SDS PAGE, as described for MVSS.

**Purity** - Absence of wild-type virus by E1 PCR, as described for MVSS. The virus production lot will be evaluated for the presence of any inapparent transforming factors or agents by cultivation on Rat 1 indicator cells (details below). The virus lot will be evaluated for the presence of residual bovine serum proteins with either a Western blot or ELISA.

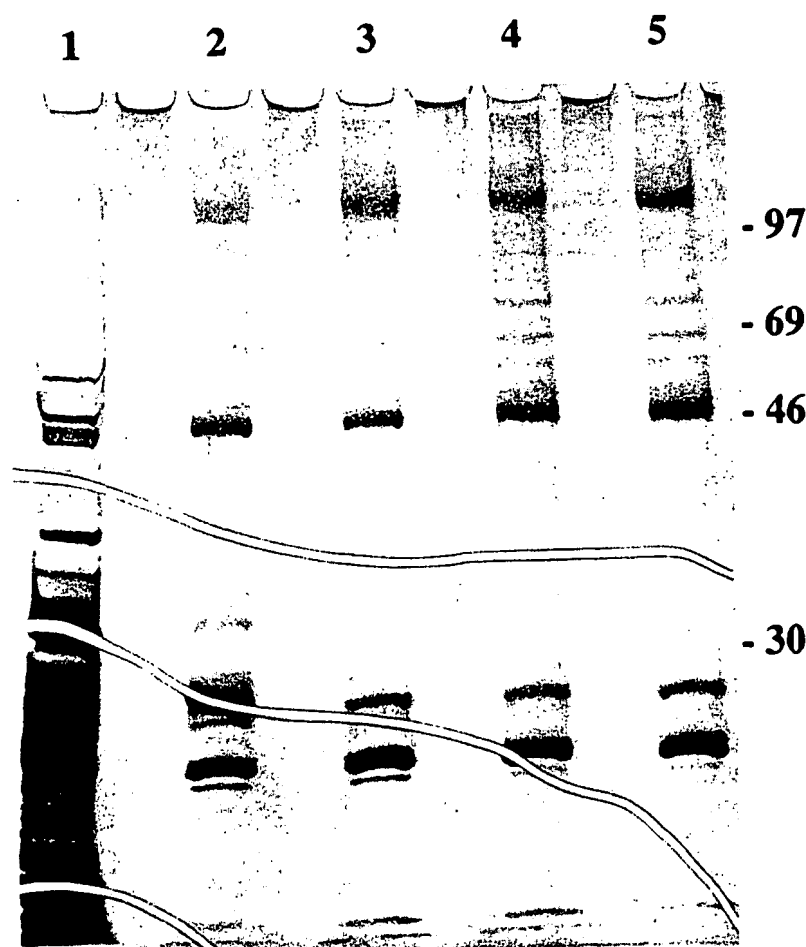
**Concentration** -  $A_{260}$ , as described above.

**Activity** - End-point CPE titration assays. From the activity and concentration measurements, we can calculate a particles per infectious unit (I.U.) ratio (I.U. as determined by end point CPE and PFU are related by a factor of 0.7). Typically the particle/PFU ratio is 350-750, only preparations with a ratio less than 500 will be used.

Safety - The virus production lots will be assessed for sterility, general safety, and endotoxin.

#### 11. Structure and Purity of Virus

SDS polyacrylamide gel electrophoresis of purified virions reveals a number of polypeptides, many of which have been identified (77). Figure 5 shows a polyacrylamide gel of a preparation of Ad2/CFTR-1 made by the method described above.



**Figure 5. Gradient Purification of Ad2/CFTR-1.**

Figure is a silver stain of a 10-20% SDS polyacrylamide gel. Lane 1, freeze/thaw supernatant; lane 2, step gradient; lane 3, first equilibrium gradient; lane 4, second equilibrium gradient; lane 5, third equilibrium gradient. Lanes 2-5 contain approximately  $10^{10}$  particles (approximately 2.5  $\mu$ g).

When preparations of virus were subjected to one or two additional rounds of CsCl centrifugation, the protein profile obtained was indistinguishable. This indicates that additional equilibrium centrifugation does not purify the virus further, and may suggest that even the less intense bands detected in the virus preparations represent minor virion

components rather than contaminating proteins. The identity of the major protein bands is presently being established by N-terminal sequence analysis.

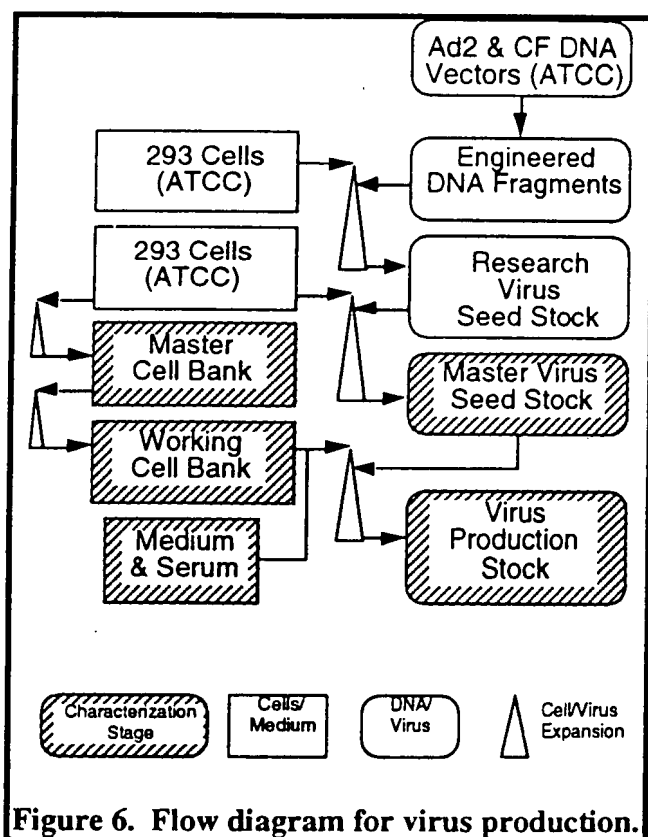
## 12. Contaminating Materials

The material to be administered to the patients will be  $2 \times 10^6$  PFU,  $2 \times 10^7$  PFU and  $5 \times 10^7$  PFU of purified Ad2/CFTR-1. Assuming a minimum particle to PFU ratio of 500, this corresponds to  $1 \times 10^9$ ,  $1 \times 10^{10}$  and  $2.5 \times 10^{10}$  viral particles, these correspond to a dose by mass of 0.25  $\mu$ g, 2.5  $\mu$ g and 6.25  $\mu$ g assuming a molecular mass for adenovirus of  $150 \times 10^6$  daltons.

The origin of the materials from which a production lot of the purified Ad2/CFTR-1 is derived was described in detail above and is illustrated as a flow diagram in Fig. 6. All the starting materials from which the purified virus is made (i.e., MCB, and WCB, and the MVSS) will be

extensively tested. Further, the growth medium used will be tested at Genzyme and the serum will be from only approved suppliers who will provide test certificates. In this way,

all the components used to generate a production lot will have been characterized. Following growth, the production lot virus will be purified by two rounds of CsCl centrifugation, dialyzed, and tested. A production lot should constitute  $1-5 \times 10^{10}$  PFU Ad2/CFTR-1.



As described above, to detect any contaminating material, aliquots of the production lot will be analyzed by SDS gel electrophoresis and restriction enzyme mapping. However, these tests have limited sensitivity. Indeed, unlike the situation for purified recombinant proteins, it is very difficult to quantitate the purity of the Ad2/CFTR-1 using SDS polyacrylamide gel electrophoresis (or similar methods). An alternative is the immunological detection of contaminating proteins (IDCP). Such an assay utilizes antibodies raised against the proteins purified in a mock purification run. Development of

such an assay has not yet been attempted for the CsCl purification scheme for Ad2/CFTR-1. However, initially we will use an IDCP assay developed at Genzyme for the detection of contaminants in recombinant proteins produced in Chinese hamster ovary (CHO) cells. In addition to hamster proteins, these assays detect bovine serum albumin (BSA), transferrin and IgG heavy and light chain derived from the serum added to the growth medium. Tests using such reagents to examine research batches of Ad2/CFTR-1 by both ELISA and Western blots are in progress.

Other proteins contaminating the virus preparation are likely to be from the 293 cells - that is, of human origin. Human proteins contaminating therapeutic agents derived from human sources are usually not problematic (121). In this case, however, we plan to test the production lot for transforming factors. Such factors could be activities of contaminating human proteins or of the Ad2/CFTR-1 vector or other contaminating agents. For the test, we propose to infect 10 dishes of Rat 1 cells containing  $2 \times 10^6$  cells (the number of target cells in the patient) with 4 times the highest human dose of Ad2/CFTR-1 ( $2 \times 10^8$  PFU). Following infection, the cells will be plated out in agar and examined for the appearance of transformed foci for 2 weeks. Wild-type adenovirus will be used as a control. Transformation assays are done routinely at Genzyme (122).

The bulk of nucleic acids and proteins would be expected to be separated from purified virus preparations upon equilibrium density centrifugation. Furthermore, we do not expect the human 293 cells to contain mouse VL30 sequences. Biologically active nucleic acids should be detected in the Rat 1 transformation assay. In addition the PCR analysis will reveal E1 sequences derived from the 293 cells.

Because only a very small proportion of a production lot will be required for human use, in excess of 95% of the preparation will be available for testing. Thus, although it is difficult to access the sensitivity of some of the assays described above, we are in a position to test samples greatly in excess of the highest human dose.

**B1.b.(1)(c) If co-cultivation is employed, what kinds of cells are being used for co-cultivation? What steps are being taken (and assays used with their sensitivity) to detect and eliminate any contaminating materials? Specifically, what test are being done to assess the material to be returned to the patient for the presence of live or killed donor cells or other non-vector materials (for example, VL30 sequences) originating from those cells?**

Not applicable.

**B.1.b.(1)(d) If methods other than those covered by (1)-(c) are used to introduce new genetic information into target cells, what steps are being taken to detect and eliminate any contaminating materials? What are possible sources of contamination? What is the sensitivity of tests used to monitor contamination?**

Not applicable.

**B.1.b.(2) Describe any other material to be used in preparation of the material to be administered to the patient. For example, if a viral vector is proposed, what is the nature of the helper virus or cell line? If carrier particles are to be used, what is the nature of these?**

The origin, growth, and characterization of the human 293 cell is described above.

**B.2. Preclinical studies, including risk-assessment studies.**  
Describe the experimental basis (derived from tests in cultured cells and animals) for claims about the efficacy and safety of the proposed system for gene delivery and explain why the model(s) chosen is (are) the most appropriate.

**B.2.a. Laboratory studies of the delivery system.**

**B.2.a.(1) What cells are the intended target cells of recombinant DNA? If target cells are to be treated *ex vivo* and returned to the patient, how will the cells be characterized before and after treatment? What is the theoretical and practical basis for assuming that only the target cells will incorporate the DNA?**

The nasal respiratory epithelium is the intended target. Cells will not be treated *ex vivo*.

The theoretical basis for assuming that only the target cells will incorporate DNA is: a) the adenovirus has a tropism for airway epithelium; b) application is to a localized area of nasal epithelium; c) the epithelium provides a barrier to virus movement from the airway lumen to the interstitial space; d) the total number of viruses applied to the surface epithelium will be very small. The practical basis for assuming that only the target cells will incorporate the DNA is that, in ongoing animal experiments, we have observed no transfer of DNA to cells other than the respiratory epithelium (Point B.2.c.(2) 3.c). However, adenovirus also has tropism for other cells such as the gut, and despite the precautions taken, we cannot be certain that no recombinant adenovirus will infect a cell other than a respiratory epithelial cell.

**B.2.a.(2) Is the delivery system efficient? What percentage of the target cells contain the added DNA?**

Because adenovirus DNA is predominantly not integrated and because the percentage of target cells that contain DNA and the copy number in those cells is dependent on the multiplicity of infection, we have not examined in detail the number of target cells that contain added DNA. Instead, we have measured the expression of the DNA; these experiments are described in response to point B.2.b.(3).

**B.2.a.(3) How is the structure of the added DNA sequences monitored and what is the sensitivity of the analysis? Is the added DNA extrachromosomal or integrated? Is the added DNA unrearranged?**

We examined the state and the copy number of Ad2/CFTR-1 DNA using Southern blot analysis. Rather than extract only soluble low molecular weight DNA by the Hirt procedure, total cellular DNA was prepared and the full complement of cellular DNA was digested with BstB1, electrophoresed and probed by Southern blotting. Figure 7 shows the state of Ad2 DNA in confluent monolayers of human primary nasal polyps cells at different times after infection with different multiplicities of Ad2/CFTR-1. The results indicate that Ad2 DNA can be detected as the predicted size BstB1 fragment from the left hand end of the genome with no evidence of high molecular weight chromosomal integrated DNA. The sensitivity of the experiment shown in Fig. 7 is not certain, but longer exposures of this and similar experiments indicate that at the 0.1-1% detection level, no integrated DNA is detected. In similar experiments we found no evidence for Ad2 DNA integration in monkey, hamster or HeLa cells at this level of detection. However, based on the literature, we would expect perhaps some integration, but at a level far below our sensitivity of detection (81,84-86).

The experiment in Fig. 7 also indicates that at high multiplicities of infection, there is time-dependent viral DNA synthesis in human nasal polyp cells. In other experiments, we have detected limited Ad2/CFTR-1 or Ad2/βGal-1 DNA synthesis in HeLa, monkey bronchiolar and hamster primary tracheal cells. We have shown that synthesis is dependent on multiplicity of infection, and found that in human cells viral DNA accumulation peaks at about 2-4 days post infection. This result was not unexpected since earlier reports of E1a- or E1b-deleted vectors reported some DNA synthesis (123-126).

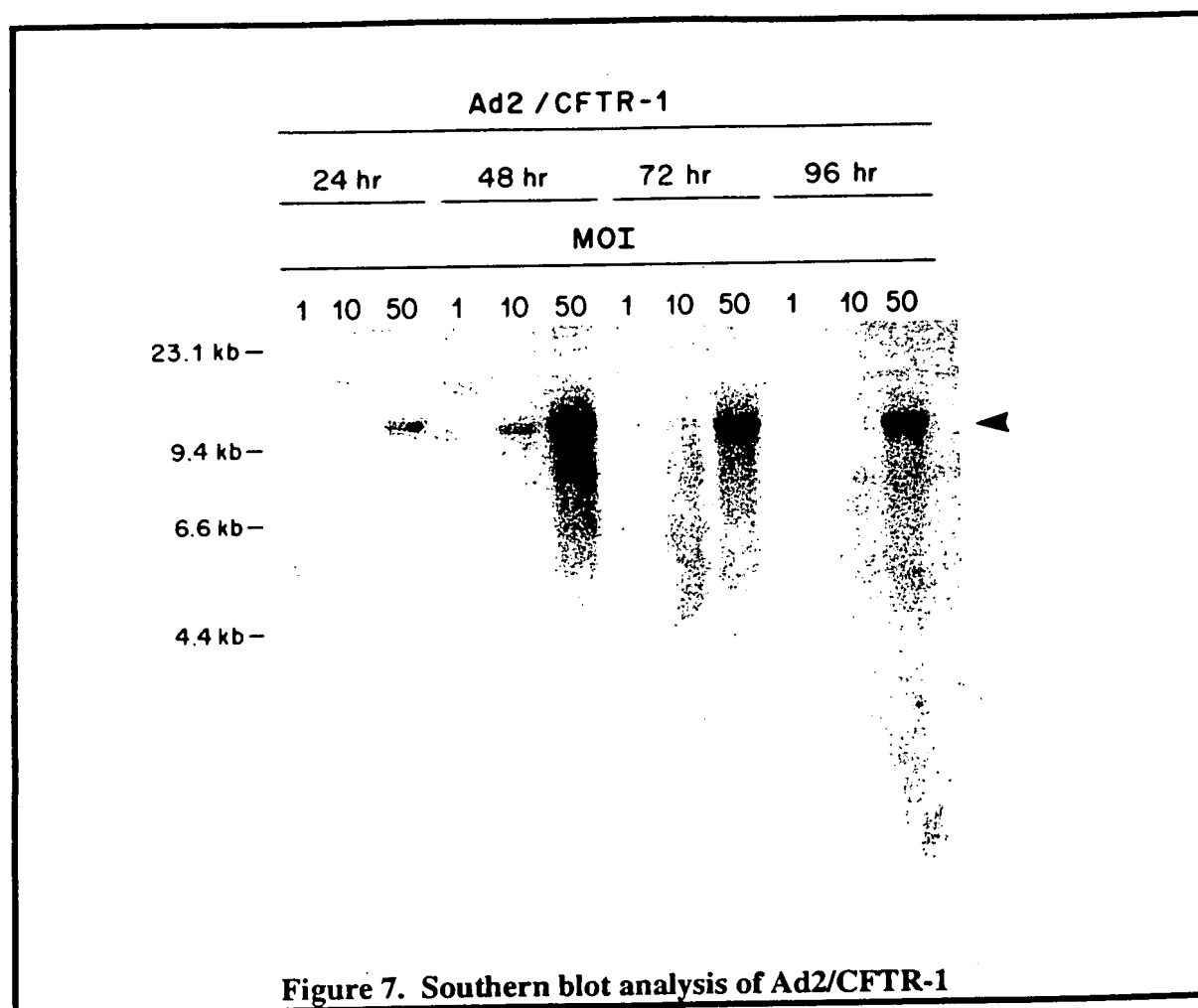


Figure 7. Southern blot analysis of Ad2/CFTR-1

The only evidence as to whether the Ad2/CFTR-1 is rearranged is the length of the viral DNA on Southern blots. Although the sensitivity of this assay is low, there is no evidence of altered molecular species indicating rearrangement.

**B.2.a.(4) How many copies are present per cell? How stable is the added DNA both in terms of its continued presence and its structural stability?**

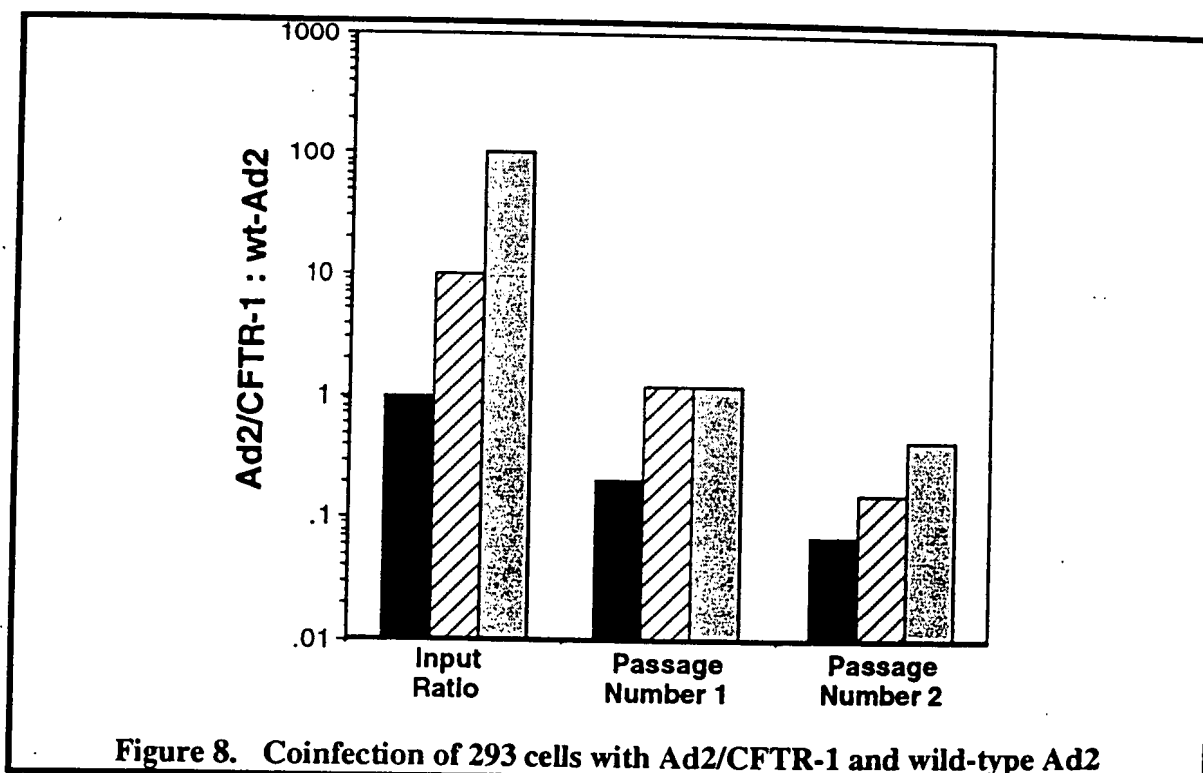
**Copy number per cell and DNA stability**

The number of copies of extrachromosomal Ad2/CFTR-DNA per cell will depend on the multiplicity of infection (MOI) used. In the proposed protocol, three different multiplicities will be tested. Because the intended area of application is about 1 cm<sup>2</sup> and the human nasal epithelium contains 2 x 10<sup>6</sup> cells/cm<sup>2</sup>, these multiplicities are 1, 10, and 25 PFU/cell. Assuming all cells take up viral DNA equally, quantitation of the Southern blot experiment using human primary nasal polyp cells grown in monolayer (Fig. 7) indicates that following infection with Ad2/CFTR-1 at an MOI of 50 the maximum number of DNA molecules detected per cell is approximately 550. In treated monkeys, we have detected  $\beta$ -galactosidase expression 2 weeks following exposure to Ad2/ $\beta$ Gal-1 and CFTR expression 1 week after exposure to Ad2/CFTR-1. There are reports in the literature suggesting transcripts from a related virus can be detected in cotton rats 42 days after exposure, using PCR methods (11). Presumably, viral DNA persists for at least this period. We do not

know the mechanism whereby DNA loss eventually occurs, but assume the unintegrated DNA is degraded by cellular nucleases.

#### Complementation and DNA stability

We assume that the limited viral DNA synthesis reported in B.2.a.(3) results from a small amount of residual viral gene expression, even in the absence of E1a and E1b gene functions. In addition, the absence of these proteins could be complemented by provision of E1 by a wild-type virus coinfecting the host cell. Under these circumstances, we expect the wild-type virus to overgrow the defective virus. Figure 8 shows an experiment which mimics such a possibility under the most favorable conditions for growth of the defective virus, i.e., in 293 cells. When the ratio of added Ad2/CFTR-1 to wild-type Ad2 was 100:1, within 2 passages the amount of wild-type virus exceeded Ad2/CFTR-1 by a ratio of 2:1.



In conditions where wild-type virus outgrows defective virus in a patient, we would expect such infection to eventually become self-limiting. Complementation of the defective virus might also occur by provision of E1 gene function as a result of an earlier, perhaps now latent adenovirus infection (100), (as a result of proteins encoded by another virus that can mimic the activity of E1 (119,127-129), or as a result of cellular proteins with E1-like activity (101,102). We have not detected growth of defective virus even in hamsters treated with adenovirus at 100 times the maximum proposed human dose or in primates treated with 2000 times the maximum proposed human dose (see below). Nevertheless, such a possibility remains. If such virus replication did occur in the patient, we would expect it to be reduced (compared to wild-type) and eventually self-limiting. As safeguards, we will test nasal cells from patients for the presence of Ad2/Ad5 E1 sequences using PCR methods prior to treatment, and will only treat patients who are seropositive for Ad2.

Finally, complementation of the growth of Ad2/CFTR-1 could occur if the preparation were infected with low levels of wild-type virus. PCR estimates of the amount of Ad2 or Ad5 E1 DNA suggest that such contamination should be below  $10^{-8}$ . Contamination at such a level could result from recombination between defective virus and the endogenous Ad5 E1 DNA sequences present in the 293 cells used to grow virus. Such a recombinant would be a Ad5E1Ad2 hybrid and eventually we would expect it to overgrow the defective virus preparation.

#### **Recombination and DNA stability**

Another way in which the adenovirus genome could be unstable would result from recombination. Homologous recombination occurs readily within adenovirus of the same subgroup (105); indeed it is routinely used in the laboratory to create variant strains. However, the products of homologous recombination would closely resemble the starting viral strains, i.e., an Ad2 recombinant with newly acquired E1 genes would essentially be a wild-type virus and a CFTR cDNA containing recombinant of the serotype C strain would be expected to have properties similar to Ad2/CFTR-1, especially a limited ability to replicate.

Non-homologous recombination could also occur but the exact structure of the products are less predictable. To be viable, the Ad2/CFTR-1 would at a minimum need to acquire E1 functions and delete other DNA sequences so as to enable packaging. The deleted sequences could involve dispensable viral genes, perhaps E3, or more likely sequences within CFTR. The activity of such recombinants is difficult to predict, but fragments of the CFTR protein seem unlikely to have activity. We have no experimental data testing for recombination, but to date we have not observed the generation of such recombinants during the routine passaging of Ad2 vectors in tissue culture cells.

#### **B.2.b. Laboratory studies of gene transfer and expression.**

##### **B.2.b.(1) What animal and cultured cell models were used in laboratory studies to assess the *in vivo* and *in vitro* efficacy of gene transfer system? In what ways are these models similar to and different from the proposed human treatment?**

##### **1. Cell lines and primary cultures**

We tested the ability of Ad2/ $\beta$ Gal-1 and Ad2/CFTR-1 to express their respective proteins in several cell lines, including human HeLa cells, human 293 cells, human pancreatic epithelial cells (CF PAC) (130), and monkey bronchiolar epithelial cells (4MBR-5). In addition, we studied primary cultures of hamster tracheal epithelial cells, cynomolgous monkey tracheal/bronchial epithelial cells, Rhesus monkey tracheal epithelial cells, and normal and CF human airway epithelia. These cells were all grown on culture dishes. Studies with these models demonstrate the ability of the vector to transfer the DNA and express the appropriate protein in epithelial cells. They are, however, different from the proposed human studies in that the cells do not form an intact epithelium.

##### **2. Primary cultures of human airway epithelia grown on permeable filter supports.**

In this model system, primary cultures of human airway epithelial cells are grown on permeable filter supports at the air-liquid interface with air on the mucosal surface (131-134). After seeding, these cells differentiate and form tight junctions to produce an electrically tight epithelial monolayer. Such cultures closely resemble the native epithelium, both morphologically (i.e., differentiated cells with tight junctions and a distinct apical and basal-lateral membrane) and functionally (i.e., low transepithelial

conductance and the capacity for vectoral transepithelial electrolyte transport). These are the characteristics which define an epithelium.

An example of their value as a model system for assessing therapy is that this system has led to the discovery and subsequent *in vivo* clinical testing of two potential therapies for CF: amiloride (17) and extracellular nucleotides (19). In addition, the ability to measure the transepithelial conductance provides the most sensitive assay of cell and monolayer integrity; with injury of epithelial cells, transepithelial conductance is an early, perhaps the first, parameter to increase (135,136).

We believe that human airway epithelia grown on permeable supports provide the model which most closely resembles human airway epithelium *in vivo*.

### 3. Nasal and bronchial epithelium of Rhesus monkeys.

This model system has the advantage of being an *in vivo* model, and it resembles the proposed human experiment. It has the disadvantage that the monkey has wild-type CFTR function; expression of recombinant CFTR on a wild-type background produces little, if any, functional evidence of CFTR expression. We have documented this by expressing CFTR in normal human airway epithelial cells; we found no additional increase in chloride secretion. Similar results have been reported by others (66). The lack of an increase in chloride secretion with overexpression of CFTR on a wild-type background likely results from limitations in transepithelial secretion resulting from rate-limiting steps occurring at the basolateral membrane (see Section 3.4). Alternatively, there could be rate-limiting steps in terms of insertion of CFTR into the apical membrane or in the signal transduction mechanisms that regulate CFTR chloride channels. As a result of these considerations, we have also used the Ad2/BGal-1 vector to assess the efficacy and safety of the delivery system. We also applied Ad2/BGal-1 to the bronchial epithelium of the monkeys to demonstrate the ability of the virus to express recombinant protein in lower airway epithelium.

### 4. CF Mice

Two groups of investigators have recently reported the development of CF mice by targeted disruption of the endogenous mouse CFTR gene (137-139). We have not yet tested Ad2/CFTR-1 in such mice. However, we would argue that such studies are not essential prior to testing Ad2/CFTR-1 in humans. a) The use of CF mice is unlikely to provide new data relevant to safety. b) The current models do not yet show lung pathology and thus do not yet provide a model of human lung disease. c) The outcome, either positive or negative, of studies directed at determining whether Ad2/CFTR-1 can express protein and restore cAMP-regulated chloride in mouse airway epithelium, would not influence our conclusions about the potential efficacy of the vector, based on our studies of efficacy in human CF airway epithelia and in primate nasal and bronchial epithelia.

#### B.2.b(2) What is the minimal level of gene transfer and/or expression that is estimated to be necessary for the gene transfer protocol to be successful in humans? How was this level determined?

As we discussed above in Section 3.3, we estimate that very little CFTR need be expressed to successfully correct the chloride transport defect in CF airway epithelia. This conclusion is based on both theoretical considerations and experimental observations.

**B.2.b.(3) Explain in detail all results from animal and cultured cell model experiments which assess the effectiveness of the delivery system (part 2.a. above) in achieving the minimally required level of gene transfer and expression (2.b. (2) above).**

**1. Cultured cell models**

**a. Studies with Ad2/βGal-1**

We infected these cells [Point B.2.b.(1)] in tissue culture and assayed for β-galactosidase activity. We used β-galactosidase tagged with a nuclear localization signal because background blue staining with X-gal is a common problem, particularly with airway cells. After exposure to Ad2/βGal-1, β-galactosidase activity was detected as a strong nuclear-localized blue staining in a variety of the different cells when infected at a multiplicity of infection between 0.01 and 30 PFUs per cell.

**b. Studies with Ad2/CFTR-1**

After exposure of these cells to Ad2/CFTR-1, we detected CFTR by immunoprecipitation followed by phosphorylation and autoradiography. We also detected CFTR by immunocytochemistry.

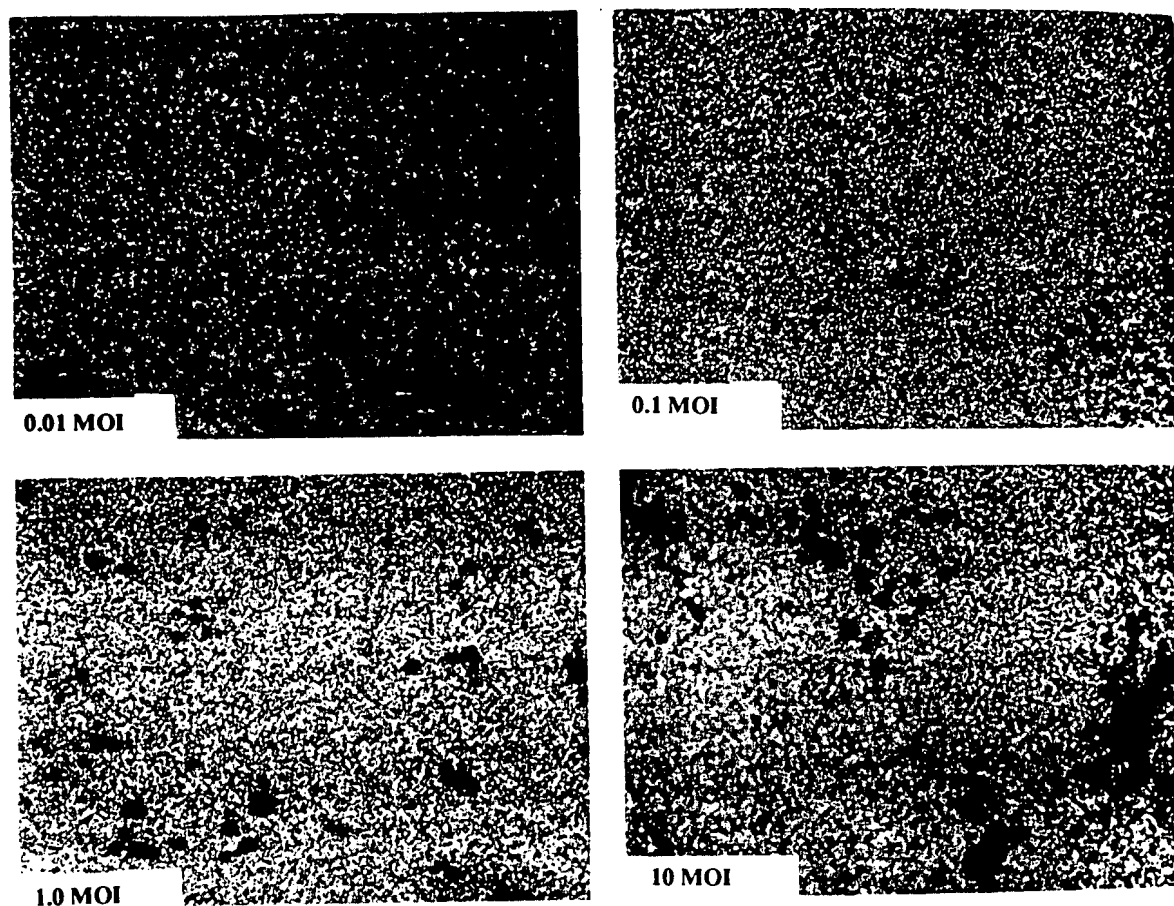
Because it is a chloride channel, CFTR can also be detected using assays based on its function. We measured cAMP-dependent CFTR chloride channels using the halide-sensitive fluorophore 6-methoxy-N-(3-sulfoethyl)-quinolinium (SPQ). In the SPQ assay, an increase in halide permeability through CFTR chloride channels results in a rapid increase in SPQ fluorescence (9). We infected CF nasal epithelial cells with Ad2/CFTR-1 at a multiplicity of 5 PFU/cell. Stimulation of Ad2/CFTR-1-infected cells with forskolin (20 μM) and IBMX (100 μM) increased SPQ fluorescence. In contrast, cAMP did not increase halide permeability in uninfected or wild-type adenovirus-infected (5 MOI) cells. Thus, only cells infected with Ad2/CFTR-1 expressed cAMP-regulated CFTR chloride channels. Some Ad-CFTR-infected cells also showed an increased basal halide permeability that was independent of cAMP stimulation. Although we have not studied this in detail, one interpretation of these data is that the adenovirus-infected cells have an elevated endogenous level of cAMP. We have obtained similar results with 293 cells. In human airway epithelial cells, we found that 15-30% of cells expressed CFTR as assessed by the acquisition of cAMP-dependent stimulation of halide permeability.

These results indicate that the adenovirus vector can direct the expression of functional CFTR chloride channels and β-galactosidase in a variety of cultured epithelial cells.

**2. Primary cultures of human airway epithelia grown on permeable supports.**

**a. Studies with Ad2/βGal-1**

To test vector efficacy, we examined the effect of Ad2/βGal-1 added at a variety of multiplicity's of infection (MOI) on primary cultures of normal human airway epithelia grown on permeable filter supports (Fig. 9). As expected, the number of stained cells depended upon the MOI; blue staining of a small percentage could be detected at an MOI as low as 0.01 PFU/cell. As the multiplicity of infection increased, so did the number of cells with blue-staining nuclei. In some cases, cells were treated with neuraminidase (0.2 U/ml) and dithiothreitol (5 mM) to remove mucus and cell debris prior to addition of virus; however, this treatment did not appear to increase the percentage of stained cells. Blue-staining cells persisted for up to 20 days after infection with Ad2/βGal-1 at 5 MOI. Longer studies were limited by the short "lifetime" of the epithelial cultures.



**Figure 9.** Effect of Ad2/βGal-1 on primary cultures of normal human airway epithelia. Epithelia were stained with X-Gal. Filters are relatively opaque, obscuring cell detail.

#### **b. Studies with Ad2/CFTR-1**

We also allowed CF airway epithelial cells grown as epithelia on permeable filter supports to form a low transepithelial conductance and then infected them with Ad2/CFTR-1. Three days after infection at 5 PFU/cell, CFTR was readily detected by immunocytochemical staining at the apical membrane (Fig. 10). In contrast, little CFTR was detected in uninfected cells or in cells infected with Ad2/βGal-1.

The most important experiment was to test the ability of Ad2/CFTR-1 to correct the defect in transepithelial chloride secretion in CF airway epithelial cells. We infected primary cultures of CF nasal polyp epithelial cells with Ad2/CFTR-1 at a multiplicity of 5 PFU/cell. Three days after infection, we measured transepithelial chloride secretion in an Ussing chamber. Figure 11 shows that CF epithelial cells do not secrete chloride upon cAMP stimulation (i.e., there is no increase in transepithelial short-circuit current with addition of cAMP). In contrast, cAMP agonists stimulated chloride secretion in Ad2/CFTR-1-treated CF epithelial cells. This response resembled that of normal nasal polyp epithelial cells and was inhibited by the chloride channel blocker, diphenylamine-2-carboxylate. We have obtained identical results using primary cultures of CF cells from tracheal epithelium.

We found that a relatively low multiplicity of infection with Ad2/CFTR-1 was able to generate apparently wild-type transepithelial chloride secretion; in fact, we detected significant chloride secretion at an MOI of 0.1. Secretion occurred at low multiplicities even though the promoter directing expression of CFTR is the E1a promoter which is not regarded as particularly strong. This result is consistent with those reported above in Section 3.3, suggesting that only modest expression of CFTR is required for phenotypic correction.

We believe that this represents the most significant data we have suggesting the efficacy of the Ad2/CFTR-1 vector. It uses human CF cells; it examines them under conditions that most closely resemble an *in vivo* epithelial monolayer; it measures the function of CFTR; and it shows that only small amounts of virus may be effective.

### 3. Primate studies.

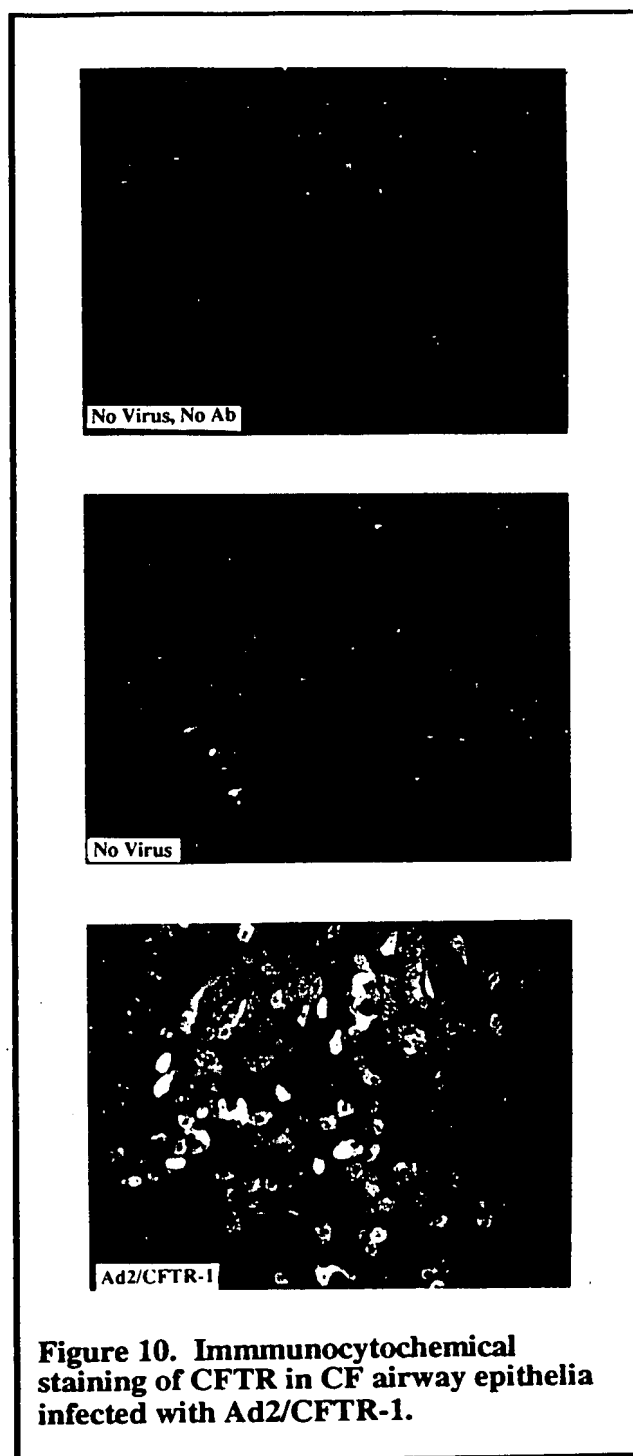
We have done three types of studies to assess the efficacy of the adenovirus constructs in the airway epithelia of Rhesus monkeys.

#### a. Ad2/βGal-1 applied to the nasal epithelium.

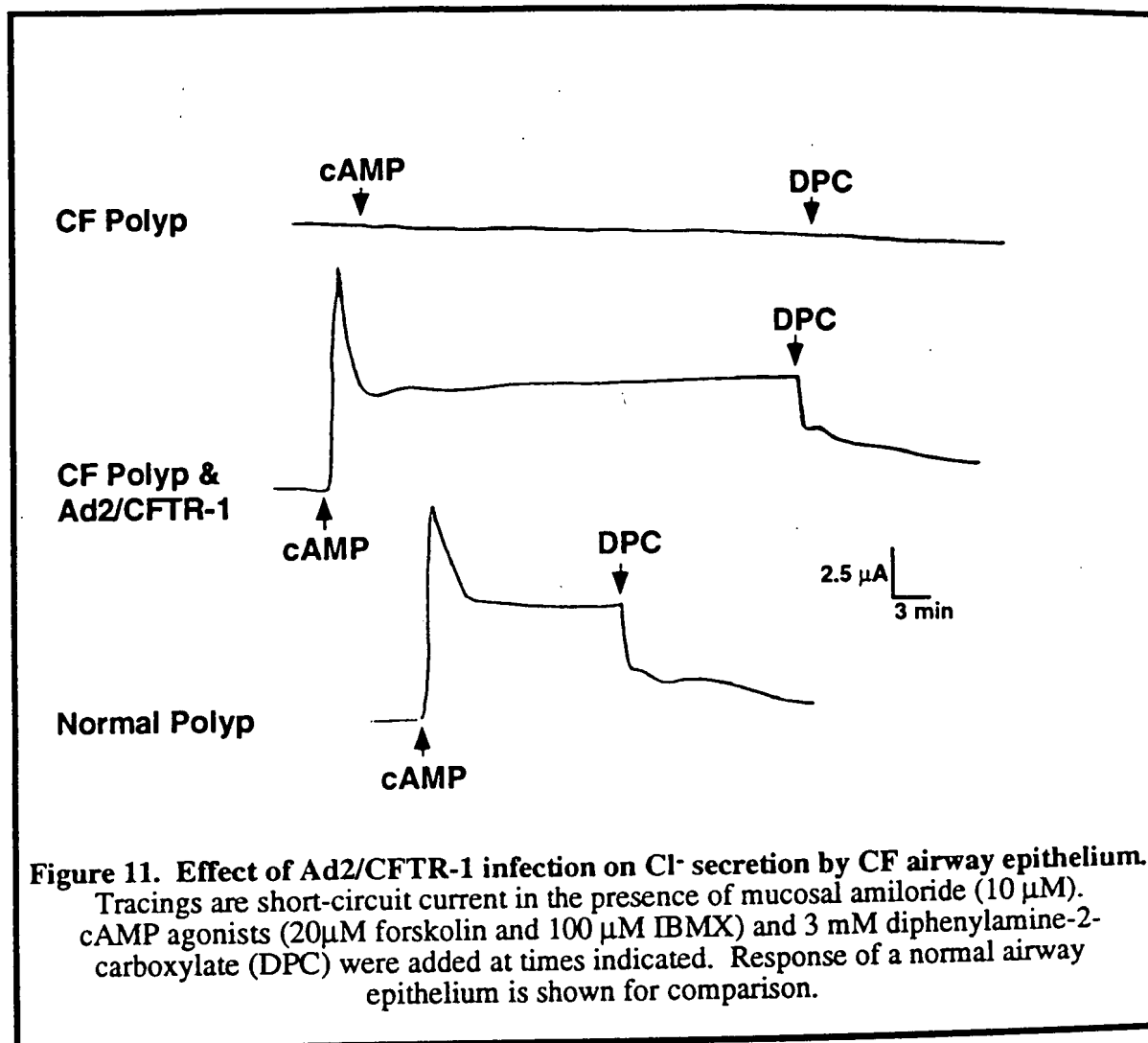
##### Procedure

For this study we have used 2 monkeys (A and B). As of late September, they have been followed for 8 weeks after virus administration.

Because primates have normal CFTR function, we elected to assess the ability of recombinant adenovirus to transfer the reporter gene for β-galactosidase by using the Ad2/βGal-1 virus. We estimated the epithelial cell density in the nasal cavity of the Rhesus monkey to be  $2 \times 10^6$  cells/cm<sup>2</sup> (based on an average nasal epithelial cell diameter of 7 μm, ref. 140) and the surface area to be 25-50 cm<sup>2</sup> (141). Thus, we estimated that there are about  $5 \times 10^7$  cells in the nasal epithelium of Rhesus monkey. Because these studies were also focused on safety, we used higher viral doses.



**Figure 10.** Immunocytochemical staining of CFTR in CF airway epithelia infected with Ad2/CFTR-1.



In our first study, we used two different preparations of Ad2/BGal-1 virus: one that was purified on a CsCl gradient and then dialyzed against tris-buffered saline to remove the CsCl, and a crude unpurified one. Titers of the Ad2/BGal-1 viruses were  $\sim 2 \times 10^{10}$  PFU in the purified preparation and  $\sim 1 \times 10^{11}$  PFU in the crude preparation. Both preparations produced  $\beta$ -galactosidase activity in 293 cells.

Monkeys were anesthetized by intramuscular injection of ketamine (15 mg/kg). We used the entire epithelium of one nasal cavity in each monkey. We inserted a foley catheter (size 10) through each nasal cavity into the pharynx, inflated it with 2-3 ml of air, and then pulled the catheter anteriorly to obtain tight posterior occlusion at the posterior choana. Both nasal cavities were then irrigated with a solution ( $\sim 5$  ml) of 5 mM dithiothreitol plus 0.2 U/ml neuraminidase in phosphate-buffered saline (PBS) for five minutes. We used this solution to dissolve any residual mucus overlaying the epithelia. (In our first *in vitro* studies with monolayers of human airway epithelia we had used a similar treatment to remove mucus. Subsequently, we have found that such treatment is not required.) The washing procedure also allowed us to determine whether the balloons were effectively isolating the nasal cavity. We then instilled the virus (Ad2/BGal-1) slowly into the right

nostril with the posterior balloon inflated. The viral solution remained in contact with the nasal mucosa for 30 minutes. At the end of 30 min, the remaining viral solution was removed by suction. The balloons were deflated, the catheters removed, and the monkey allowed to recover from anesthesia. Monkey A received the CsCl<sup>-</sup> purified virus and Monkey B received the crude virus.

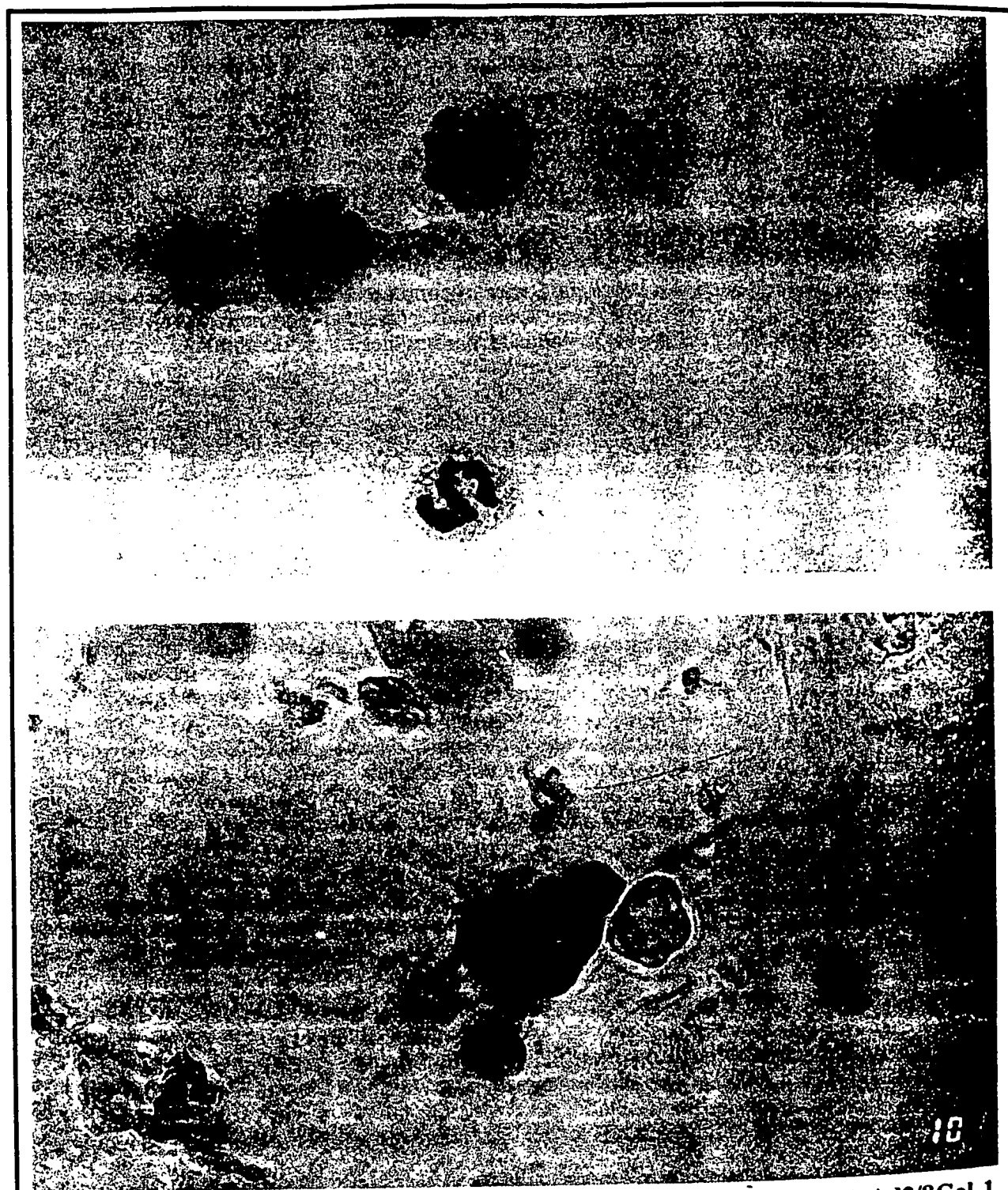
To obtain nasal epithelial cells from an anesthetized monkey, we first impregnated the nasal mucosa with 5 drops of Afrin (0.05% oxymetazoline hydrochloride, Schering-Plough) and 1 ml of 2% Lidocaine for 5 min. We then used a cytobrush (the kind typically used for Pap smears) to gently rub the mucosa for about 3 seconds. For tracheal brushings, we used a flexible fiberoptic bronchoscope; a 3 mm cytology brush (Bard) was advanced through the bronchoscope into the trachea and a small area was brushed for about 3 seconds. We repeated this procedure twice to obtain a total of  $\sim 10^6$  cells. To obtain pharyngeal epithelia, we rubbed a cotton-tipped applicator over the back of the pharynx 2-3 times. The resulting cells were dislodged from brushes or applicators into 2 ml of sterile PBS.

### Results

To detect vector-generated mRNA we used reverse transcriptase PCR (RT-PCR). We used PCR primers in both the adenovirus sequence and the Lac Z sequence. RT-PCR of brushed cells revealed a band of the correct size that hybridized with a  $\beta$ -Gal probe, consistent with the presence of  $\beta$ Gal mRNA in the samples from both monkeys. RT-PCR was positive on days 3 and 7 and was also positive with cells from the control nostril of monkey A on day 3.

We also confirmed the presence of mRNA by using *in situ* hybridization to detect transcripts in nasal epithelial cells obtained by brushing. At day seven after infection, we observed specific hybridization with the antisense, but not the sense, probe in cells from the right nostril. Only a rare positive cell was detected from the left (control) nostril.

Cells obtained by nasal brushing were also examined for  $\beta$ -galactosidase activity by staining with X-Gal. X-Gal stains revealed blue-stained cells with clear nuclear localization of the pigment through day 7 post infection. X-Gal stains from the right nostril of monkey A, revealed approximately 1% of cells stained with nuclear-localized blue stain; these cells were predominantly ciliated respiratory cells. Figure 12 shows an example of the ciliated respiratory epithelial cells obtained by brushing (Wright stain) and shows cells with blue-stained nuclei. Monkey B had a lower percentage of stained cells from the infected nostril and none from the control side. Blue cells were not observed on day 14 or in subsequent samples.



**Figure 12.** Cells obtained by brushing monkey nasal epithelium infected with Ad2/BGal-1. Top panel shows cells stained with Wright stain (630X magnification); ciliated epithelial cells are readily apparent. Field also contains a neutrophil. Although this was the only neutrophil on the entire slide, it is included to show the ability of nasal brushings to detect inflammatory cells. Bottom panel shows cells stained with X-Gal (400X magnification).

We also tested for the presence of Ad2/βGal-1 DNA by PCR of the brushed samples. Although the results do not reveal whether the virus was transcribing β-galactosidase mRNA or producing β-galactosidase protein, the test is the most sensitive assay for the presence of viral DNA. Moreover, we expect that some of the viral DNA detected by PCR will be generating protein below the threshold of detection of the tests employed. We used the same primers used for the RT-PCR. After 2 weeks, DNA was detected only from the right (experimental) nostril. Viral DNA encoding β-galactosidase was no longer detected 4 weeks after virus application.

### Conclusions

The results of this study showed that a gene can be expressed by a recombinant adenovirus *in vivo* in primate respiratory epithelial cells. In the absence of data on the sensitivity of the X-gal staining procedure or on quantitation of the PCR reaction, it is difficult to estimate the number of nasal cells that took up the Ad2/βGal-1 vector DNA. We also found the transient presence of β-galactosidase protein and mRNA in the control nostril and in the pharynx. We think the virus most likely reached the contralateral nostril and pharynx via drainage by gravity after we released the posterior occlusion or it may have been caused by the monkey putting its finger from one nostril to another after recovery from anesthesia.

### b. Ad2/CFTR-1 applied to the nasal epithelium.

#### Procedure

For this study, we used 3 monkeys (C, D, & E). As of late September, they had been followed for 4 weeks after virus application.

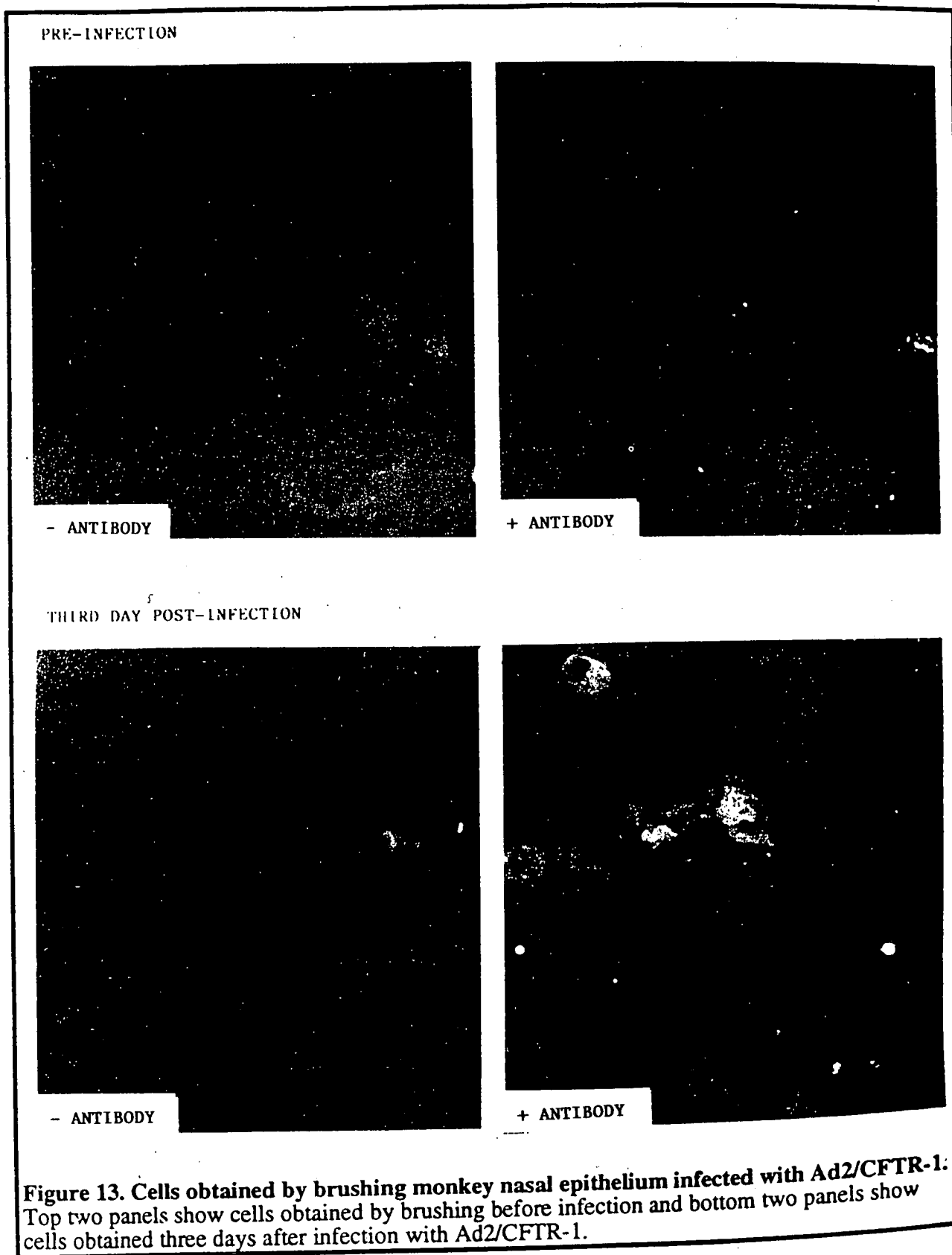
We applied the Ad2/CFTR-1 construct to the nasal epithelium exactly as described above for studies with the Ad2/βGal-1 virus, with the exception that the epithelium was not pretreated with dithiothreitol and neuraminidase. In each monkey, we applied  $2.5 \times 10^9$  PFU in 0.4 ml of CsCl purified, dialyzed virus to the right nostril. Airway epithelial cells were obtained by brushing, as described above.

#### Results

RT-PCR of brushed cells was positive on day 3 and 6 on the right, but not the left nostril. After infection RT-PCR (using primers in the adenovirus sequence and the CFTR sequence) revealed a band of the correct size which hybridized with a CFTR probe, consistent with recombinant CFTR mRNA in the samples.

Cells were also processed on cytospin slides and prepared for immunocytochemistry using monoclonal antibodies to CFTR. On day 3 after infection, positive immunofluorescence was detected in all three monkeys, predominantly in cells from the right nostril, although a few cells stained positive from the left, control nostril. Figure 13 shows an example. CFTR was also detected by immunostaining in two of the monkeys at one week after infection. CFTR immunofluorescence was negative in samples from monkeys receiving Ad2/βGal-1. (Note, that we have not determined whether our antibodies to human CFTR crossreact with endogenous monkey CFTR.)

PCR of CFTR DNA encoded by Ad2/CFTR-1 was positive in cells from the right nostril, but not the left nostril or pharynx, for two weeks after infection in all of the monkeys.



## Conclusion

The results of these studies indicate that the Ad2/CFTR-1 virus directs expression of CFTR mRNA and protein *in vivo* in primate respiratory epithelial cells. As in the study with the Ad2/βGal-1, we found that there was some cross contamination of the control nostril; again, this most likely is a result of physical movement of the virus from one side to the other. As with Ad2/βGal-1, we were unable to quantitate expression of CFTR in monkey nasal cells since we are unsure of the sensitivity of the immunocytochemical assay. It should be noted, however, that using the same antibody to detect CFTR in normal human airway tissue endogenous protein is exceedingly difficult to detect with certainty (32).

### c. Ad2/βGal-1 applied to the bronchial epithelium.

#### Procedure

For this study, we used 2 monkeys (G & H). As of late September, they have been followed for 5 weeks.

The monkeys were anesthetized by intramuscular injection of ketamine (15 mg/kg) and 0.5 mg of atropine. Flexible fiberoptic bronchoscopy was performed after instilling 1 ml of 1% lidocaine over the vocal cords. An airway lavage catheter (Baxter 792017, a modified pulmonary artery catheter with two balloons separated by 15 mm and two ports that end between the two balloons), was inserted through the bronchoscope channel. The monkeys were placed in the left lateral decubitus position to decrease shunt, and the distal balloon was inflated with 2 ml of air at the level of the take off of the right middle lobe. The proximal balloon was then inflated below the take off of the right upper lobe. This procedure isolated a small area of the bronchus intermedius. Ad2/βGal-1 ( $1.6 \times 10^8$  PFU in 0.5 ml) was then instilled through one of the inter-balloon ports and flushed with 0.25 ml of tris-buffered saline (TBS). After 15 min the balloons were deflated, allowing the Ad2/βGal-1 virus preparation to run by gravity into the right middle lobe and the right lower lobe. The bronchoscope was then removed and the monkeys recovered from anesthesia.

To obtain bronchial epithelial cells, the monkeys were anesthetized as described above, and flexible fiberoptic bronchoscopy was performed. A 3 mm cytology brush (Bard) was advanced through the bronchoscope and a small area of the bronchus intermedius and the left main bronchus was brushed gently for about 3 seconds. The resulting cells were dislodged from brushes into 2 ml of sterile PBS. Bronchoalveolar lavage was performed by wedging the tip of the bronchoscope in the right middle lobe bronchus. An aliquot of 20 ml of sterile PBS at room temperature was infused. Fluid was then withdrawn by hand suction into a syringe. The return from the 20 ml infusion ranged between 8 and 14 ml.

#### Results

The cell suspension was processed as described above in 3.a. X-Gal stains failed to reveal any blue cells at any point of the study. We believe this may have been due to the low sensitivity of the chromogenic staining technique. Therefore, for the samples from day 14, we elected to use a fluorescent assay, employing FDG (fluorescein di-β-D-galactopyranoside, Molecular Probes Inc) as the reporter for the presence of β-galactosidase activity. The use of FDG in a fluorescence-activated cell analysis is reported to be several orders of magnitude more sensitive than chromogenic assays (142). When analyzed by a fluorescence-activated cell sorter, bronchial epithelium from Monkey H revealed a bimodal distribution of fluorescence intensity consistent with β-galactosidase activity in at least 10% of the cells. No β-galactosidase activity was detected in bronchial

epithelial cells by day 21 post infection in Monkey H. No  $\beta$ -galactosidase activity was observed in a similar preparation from a control monkey (Monkey F, below).

PCR to test for the presence of Ad2/ $\beta$ Gal-1 DNA in the brushed samples was positive in the right bronchus and bronchoalveolar lavage samples from the right side for 7 days.

### **Conclusions**

These studies show that recombinant adenovirus can target the bronchial epithelium, as well as the nasal epithelium, and direct protein expression. Using a more sensitive assay method we detected enzyme activity in at least 10% of cells 14 days after treatment. No activity was detected in control animals nor in treated samples taken at later times.

### **B.2.b.(4) To what extent is expression only from the desired gene (and not from the surrounding DNA)? To what extent does the insertion modify the expression of other genes?**

Since we have no evidence for integration of adenovirus DNA into the host chromosome, we do not expect major changes in the expression of host cell DNA resulting from treatment of cells or monolayers with Ad2/CFTR-1.

### **Host cell shutoff**

In the course of a normal adenovirus productive infection cycle, host cell macromolecular synthesis is inhibited (host cell shut-off) (123). This process is known to require E1b gene function and consequently, we do not expect treatment with Ad2/CFTR-1 to lead to host cell shut-off. Perhaps the best evidence that this does not occur is the finding that Ad2/CFTR-1 treated CF nasal polyp monolayers that express CFTR as evidenced by phenotypic correction are viable for at least 2-3 weeks in culture. Presumably, even if some shut off does occur, it has no deleterious effect on long-term cell metabolism.

### **Viral gene expression**

Reports in the literature indicate that viruses deleted for E1a or E1b have a limited ability to reproduce (101,102,123-126) and in Fig. 8 we showed evidence for limited DNA synthesis by Ad2/CFTR-1 in human cells. This implies that, although reduced, expression of viral genes is not completely inhibited especially at high MOI. Indeed, it was for this reason that we left intact the E3 region. We therefore examined expression of early and late viral genes by Northern Blot analysis using as controls either wild-type or defective plus wild-type helper infected cells. We detected transient, dose-dependent expression of E4 and L5 transcripts in HeLa and CFPAC cells. The levels of expression were greatly reduced compared with wild-type infected cells (less than 0.1%). We have not attempted to detect viral proteins encoded by these mRNAs, but again, our experience with human nasal polyp monolayers suggests that no toxicity is associated with this limited viral gene expression.

### **B.2.b.(5) In what percentage of cells does expression from the added DNA occur? Is the product biologically active? What percentage of normal activity results from the inserted gene?**

It is difficult to be certain of the exact percentage of cells that express the DNA, but the studies with the CF airway epithelia cultured on permeable filter supports suggest that it is possible to complement the CF chloride transport defect at an MOI of 5 PFU/cells and that it is possible to obtain detectable cAMP-stimulated chloride secretion with as little as 0.1 PFU/cell.

**B.2.b.(6) Is the gene expressed in cells other than the target cells? If so, to what extent?**

We have not examined CFTR expression in cells other than the airway or nasal epithelium in animals treated with Ad2/CFTR-1.

A number of studies have shown that functional CFTR chloride channels can be expressed in a wide variety of cells in culture without obvious deleterious effects (9,23,25,29,33-36,38,66). Were Ad2/CFTR-1 to infect human cells other than the target, it is possible that the E1a promoter would be active and CFTR would be expressed. We have argued above, Section 3.4, that because the activity of CFTR is tightly regulated, expression of the protein need not necessarily lead to alterations in overall ion transport or cell function. We also described transgenic animals expressing very high levels of CFTR in lung (71) and mammary glands (72) in which no deleterious effects were seen. The same conclusion was drawn from preliminary examination of transgenic animals expressing CFTR under the influence of the ubiquitous  $\beta$  actin promoter.

**B.2.c. Laboratory studies pertaining to the safety of the delivery/expression system.**

**B.2.c.(1) If a retroviral system is used: (a) - (e)**

These questions do not apply to our protocol.

**B.2.c.(2) If a nonretroviral delivery system is used: What animal studies have been done to determine if there are pathological or other undesirable consequences of the protocol (including insertion of DNA into cells other than those treated, particularly germ line cells)? How long have the animals been studied after treatment? What tests have been used and what is their sensitivity?**

Studies of the adenovirus constructs have been performed with Syrian hamsters and primates. In addition, safety aspects are addressed with studies of virus-treated cell lines and studies of primary cultures of human airway epithelia grown on permeable supports.

**1. Cultured Cells**

The consequences of administering Ad2/CFTR-1 or Ad2/BGal-1 to cultured cells were described above. These studies provided evidence for limited early and late viral gene expression and limited transient viral DNA synthesis.

As a follow up to these results, we have in some cases examined virus replication by the ability of culture supernatant from Ad2/CFTR-1 treated cells to plaque or to cause CPE in 293 cells. Such experiments reveal no evidence for Ad2 vector replication, presumably because even if limited gene expression occurs, insufficient protein is produced to support assembly of progeny virions. More importantly, we also have no evidence of vector replication from animal experiments as described below.

On some occasions we have detected CPE on 293 cells in assays of Ad2 vector-treated human cell supernatants. When characterized by restriction enzyme analysis, only wild-type Ad2 virus DNA was detected. We do not know the origin of the wild-type virus, it could have originated in the original human cells themselves, been present at the low level in the input virus, or, perhaps most likely, resulted from contamination within the laboratory.

## 2. Primary cultures of human airway epithelia grown on permeable supports.

### Procedures

As described in Point B.2.b.(3), normal and CF airway epithelia were cultured on permeable filter supports and allowed to develop a low transepithelial conductance. They were then exposed to either the Ad2/CFTR-1 or the Ad2/βGal-1 virus.

### Results

The transepithelial conductance of normal and CF airway epithelial monolayers infected with Ad2/CFTR-1, Ad2/βGal-1, or no virus were indistinguishable. Figure 14A shows the time course of transepithelial conductance from 4 CF monolayers studied for 9 days after application of either Ad2/CFTR-1 (5 MOI) or vehicle control; Fig. 14B shows mean data from 7 control CF epithelial monolayers and 10 Ad2/CFTR-1-treated (5 MOI) monolayers

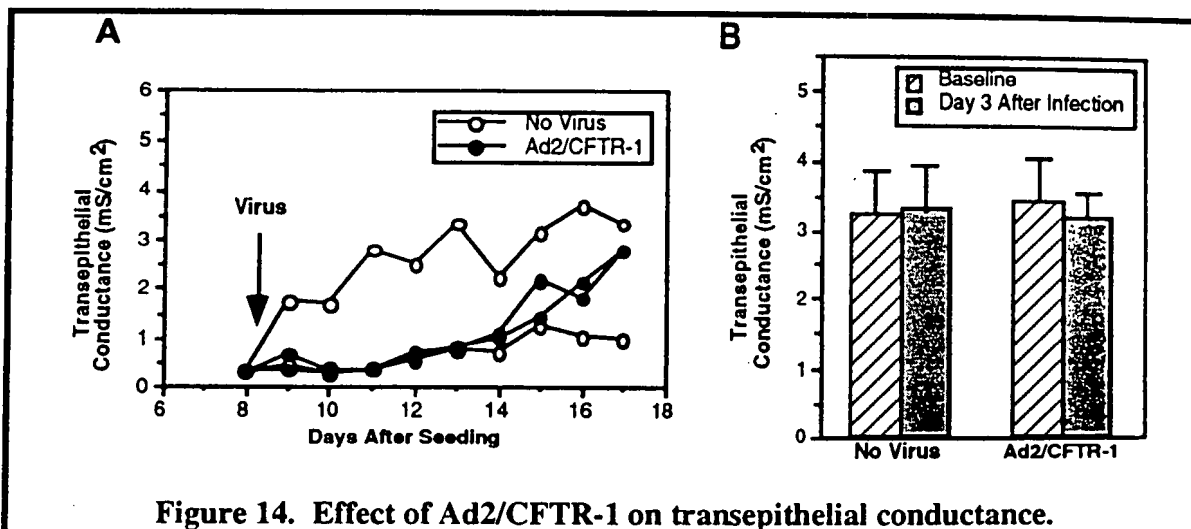


Figure 14. Effect of Ad2/CFTR-1 on transepithelial conductance.

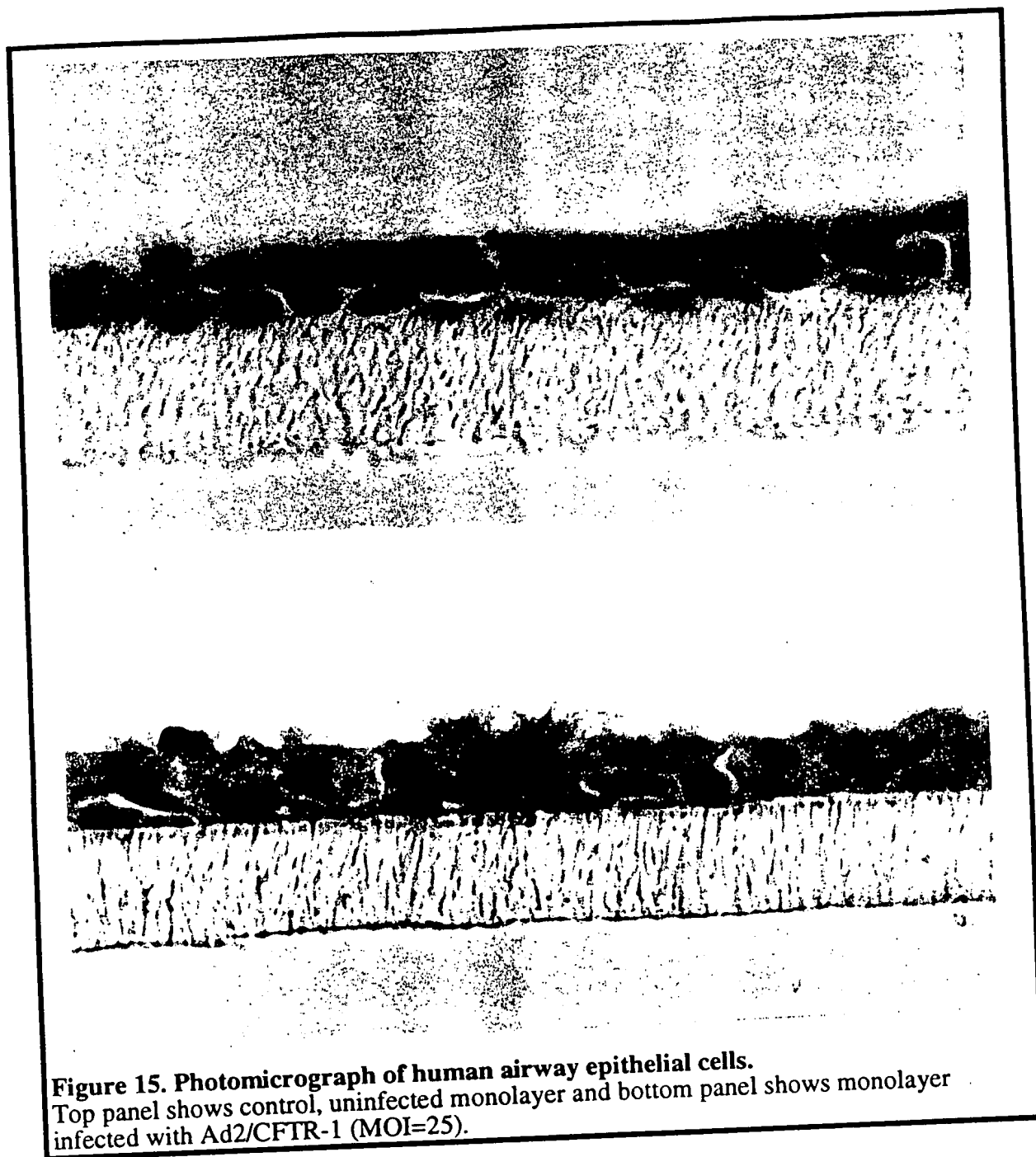
followed for 3 days after infection. The data indicate that there was no significant effect of virus on transepithelial conductance; transepithelial conductance usually begins to increase after two weeks in culture, because the monolayers have a limited lifetime. Moreover, infection with either of the two recombinant viruses did not alter the electrolyte transport properties (amiloride-sensitive Na<sup>+</sup> absorption and cAMP-stimulated chloride secretion). When the infected and uninfected monolayers were compared by light microscopy, they were indistinguishable; Fig. 15 shows an example.

### Conclusion

These data indicate that infection of epithelial monolayers with the recombinant adenoviral vectors did not produce any adverse effects on tight junctions, cell morphology, or the capacity for transepithelial electrolyte transport. The lack of alteration in transepithelial conductance, which is directly related to the permeability of the epithelial barrier, indicates that there was no effect on the integrity of the epithelium.

## 3. Hamster studies.

Several studies involved the intratracheal instillation of the Ad2/βGal-1 viral vector into Syrian hamsters. The hamster is a permissive host for serotype C adenovirus (143,144) and has been used as a model system.



**Figure 15. Photomicrograph of human airway epithelial cells.**  
Top panel shows control, uninfected monolayer and bottom panel shows monolayer infected with Ad2/CFTR-1 (MOI=25).

#### a. Study One.

##### Procedures

This study was a time course assessment of the pulmonary and systemic acute inflammatory response to a single intratracheal administration of Ad2/βGal-1. In this study, a total of 24 animals were distributed among three treatment groups: 8 vehicle control, 8 low dose virus ( $1 \times 10^{11}$  particles;  $3 \times 10^8$  PFU), and 8 high dose virus ( $1.7 \times 10^{12}$  particles;  $5 \times 10^9$  PFU). Within each treatment group, 2 animals were analyzed at each of four time points after viral vector instillation; the times were 6 hrs, 24 hrs, 48 hrs, and 7

days. At the time of sacrifice of each animal, lung lavage and blood samples were taken for analysis. The lungs were fixed and processed for normal light-level histology.

Blood and lavage fluid were evaluated for total leukocyte count and leukocyte differential. As an additional measure of the inflammatory process, lavage fluid was evaluated for total protein. Lung lavage fluid was also evaluated for the presence of infectious viral particles. Following embedding, sectioning and hematoxylin/eosin staining, lung sections were evaluated for signs of inflammation and airway epithelial damage.

### Results

With the small sample size, the data from this preliminary study were not amenable to statistical analyses, however, some general trends could be ascertained. In the peripheral blood samples, total leukocyte counts showed no apparent dose- or time-dependent changes. In the blood leukocyte differential counts, there was a minor dose-related elevation in percentage of neutrophils at 6 hours, however, data from all other time points showed no elevation in neutrophil percentages. Taken together, these data suggest a minimal and transient systemic inflammatory response to viral administration.

From the lung lavage, some elevation in total neutrophil and percentage neutrophil counts were observed at the first three time points (6 hr, 24 hr, 48 hr). By seven days, both total and percentage of neutrophil values had returned to the normal range. The trends in lung lavage protein levels were more difficult to assess due to inter-animal variability, however, no obvious dose- or time-dependent effects were readily apparent. From the lung histology, two clear observations were apparent. First, no damage to airway epithelium was observed at any time point or virus dose level. Second, a time- and dose-dependent mild inflammatory response was observed, being maximal in the 48 hr high virus dose animals. By seven days, the inflammatory response had completely resolved, such that the lungs from animals in all treatment groups were indistinguishable.

In the virus titer assay of the lung lavage fluid, cytopathic effect (CPE) was observed in one of two control animals from the 6 hr and 24 hr time points, and in one of two high dose animals at all four time points. Culture supernatants from the initial CPE positive plates were reinoculated onto 293 cells, and examined by immunofluorescence for adenovirus structural proteins. DNA was extracted and characterized by restriction enzyme analysis. Only two of the CPE positive samples stained with adenovirus antibody (6 hr high dose, 48 hrs high dose) and of these the 6 hr sample has been confirmed as Ad2/βGal, presumably residual input virus. No sample has been confirmed to have wild-type virus. We assume that the majority of samples giving CPE, contained other viruses or agents, perhaps originating from the hamsters.

### Conclusion

In summary, a mild, transient, inflammatory response appears to be associated with the intratracheal administration of the described doses of adenoviral vector in the Syrian hamster. Of note, the high dose hamsters received 100 times the number of viral PFUs as we propose to deliver to the highest dose human.

### b. Study Two.

#### Procedures

A single intratracheal Ad2/βGal-1 administration hamster study is in progress. This study is designed to assess the possibility of productive infection of organs outside of the lung, the possibility of a long term pulmonary inflammatory response, and the antibody response of the animals to the adenoviral vector. In this study, the three treatment groups are:

vehicle control, low dose virus ( $6 \times 10^6$  PFU), and high dose virus ( $8 \times 10^7$  PFU). Each group contained 9-12 animals. The doses expressed as particles per animal were low dose,  $3.5 \times 10^{11}$  and high dose  $5 \times 10^{12}$ . The particle to PFU ratio of this preparation of virus was lower than usual; we noted aggregation upon purification of this preparation, perhaps because of the scale required for this preparation of virus. Two to four animals per virus dose level were evaluated at three time points: 1 day, 1 week, and 4 weeks. In this study, viral vector persistence and possible spread is being evaluated by the assessment of the presence of virus in numerous organs including lung, gut, heart, liver, spleen, kidney, brain and gonads. The presence of virus in the various tissues is being evaluated by CPE assay of tissue homogenates where possible and, where tissue homogenates interfere with such assays, indirect immunofluorescence detection of viral proteins is being used. Adenoviral antibody titer has been measured in peripheral blood and lung lavage samples. Additionally, lung lavage, peripheral blood and lung histology were evaluated as in the previous study.

### Results

The first phase of this study is completed, and evaluation of the large number of samples is ongoing. In the peripheral blood, total leukocyte counts were, in general, within normal range. A mild to moderate leukopenia was observed in several animals of all three treatment groups at the 4 week time point. However, this leukopenia did not appear to be a dose-related phenomenon. The only consistent dose-related effect observed was a mild elevation in total leukocyte count in the 1 week animals which had received the high dose of virus. Blood leukocyte differential counts were also generally normal throughout the study. Several animals in each treatment group had a mildly elevated percentage of neutrophils at the 1 day time point. Again, this observation did not appear to be related to virus dose. Neutrophil percentages were within the normal range for all treatment groups at both 1 and 4 weeks.

In the lung lavage samples, no dose-dependent alterations in either total leukocyte count or leukocyte differential were observed. A few animals from all groups at the 1 day time point showed a slightly elevated percentage of neutrophils, consistent with an instillation-associated mild inflammatory response. Lavage samples from animals at all time points and treatment groups also showed some level of erythrocytes, both free and within phagocytic cells. The cause of this observation is not clear, however, it does not appear to be treatment-related. The absence of dose-related adverse effects on the lungs was also confirmed by the lavage protein data. There were no dose- or time-related changes in lung lavage protein observed. Although two animals from the 1 week control group exhibited moderately elevated protein levels, all other 1 week animals had normal protein levels in the lavage fluid. The histological evaluation of the lungs was also consistent with the observations noted above. Specifically, no dose- or time-related changes in lung histology were observed; airway epithelium was intact and no focal or widespread inflammation was observed.

The lung lavage samples from 1 day and 1 week, and fecal samples from 1 week time points of all three treatment groups have been tested for the presence of infectious viral particles. No infectious virions were detected. The one month lung tissue samples from all four of the high dose and lung lavage samples from two high dose animals have also been tested and again no infectious virions detected. Remaining lavage, lung tissue and fecal samples from other time points are currently being tested.

A clear time and dose-dependent serum antibody response was detected in all the hamsters. It should be noted that as expressed as mass/kg these animals were treated with approximately 50,000 times the maximum human dose proposed in our study. Lung lavage

samples from the 4 week animals also showed evidence of a dose-dependent increase in Ad2 antibody titer but at approximately a 100 times lower level than in serum.

### **Conclusions**

The intratracheal administration of the described doses of Ad2/βGal-1 into hamsters does not appear to cause any persistent pulmonary or systemic adverse inflammatory events. Additionally, infectious virions were not detectable in either lung lavage or feces of the 1 week animals nor in one month high dose lung tissue samples.

### **c. Studies in progress**

A separate study, designed specifically to focus on detection of viral DNA in various organs to assess the spread of added virus is in progress. In this study, male and female Syrian hamsters have been given Ad2/βGal-1 ( $1.4 \times 10^{10}$  PFU) by intratracheal instillation. At 7 days post-instillation, various tissues, including lung, gut and gonads, were removed and analyzed by PCR for the presence of Ad2/βGal-1 DNA. Under conditions where vector DNA was readily detected in treated lung samples, we have observed no confirmed PCR signal in liver, kidney, ovary and testes, nor in the lungs of sentinel hamsters. This experiment is being repeated with other species and other tissues, but results so far suggest that no transmission of vector DNA to other tissues, including the gonads, has occurred.

An additional study, utilizing wild-type Ad2 is also in progress. The objectives of this study are twofold, to confirm the reported susceptibility of Syrian hamsters to wild-type adenovirus serotype C infection (91,144), and to provide positive control tissues for both immunofluorescence localization of adenoviral protein within various tissues and organs. Six male Syrian hamsters will be given intranasal doses of  $1 \times 10^7$  PFU of wild-type Ad2 virus. At 3, 5 and 7 days after virus administration, two animals will be sacrificed and various organs harvested for analysis. Lung and blood samples will be analyzed for the presence of infective virions. Other organs (as listed in the previous study) will be analyzed by immunofluorescence localization methods for the presence of viral proteins.

To improve sample size, an additional study, similar in design to study one, but using larger numbers of animals, will begin shortly. We are also repeating the experiment in cotton rats, another host permissive for adenovirus replication. Both of these studies will use Ad2/CFTR-1.

### **Conclusions**

Taken together, these hamster data suggest little or no systemic inflammatory response to the viral administration. There was a mild, transient, pulmonary inflammatory response which appeared to be associated with the intratracheal administration of the described doses of adenoviral vector in the Syrian Hamster. Serum antibody to adenovirus was detected in all treated animals. No evidence of vector replication was obtained.

## **4. Primate studies.**

### **a. Ad2/βGal-1 applied to the nasal epithelium.**

#### **Procedure**

The procedure for two monkeys (A and B) was described above, point B.2.b.(3). The monkeys were completely evaluated on days 1, 4, 7, 14, 21, 28, 42, and 63. The monkeys were also observed daily for any abnormal behavior or physical signs.

## Results

*Examination.* Both monkeys tolerated the procedure well. Visual inspection of the nasal epithelium revealed no inflammatory response. Daily examination revealed no evidence of coryza or conjunctivitis. There was no cough, sneezing, or diarrhea. For both monkeys, the nasal mucosa was mildly erythematous in both the infection side and the control side on the first day after infection; we interpret this as a change due to the instrumentation. This decreased in subsequent studies as our techniques improved. Appetites and weights were not affected by virus administration in either monkey. Physical examination revealed no evidence of lymphadenopathy, tachypnea, or tachycardia. On day 21, monkey B had a temperature  $39.1^{\circ}\text{C}$  (normal for Rhesus monkey  $38.8^{\circ}\text{C}$ ) but had no other abnormalities on physical exam or in laboratory data.

*Blood counts and serology.* Monkey A had a slight leukocytosis on day 1 post infection which returned to normal by day 4: the WBC was 4,920 on the day of infection, 8,070 on day 1, and 5,200 on day 4. The ESR, serum electrolytes, transaminases, BUN and creatinine were normal throughout.

*Cytology of the nasal epithelium.* We assessed epithelial inflammation by cytological examination of Wright-stained cells (cytospin) obtained from brushings of the nasal epithelium. We compared the percentage of neutrophils and lymphocytes to that of the control nostril and to the normal values from four control monkeys. Wright stains of cells from nasal brushing were performed on each of the evaluation days. They revealed less than 5% neutrophils and lymphocytes. There was no difference between the infected and the control side.

*Presence of virus and virus replication on the airway epithelium.* We tested for the presence of virus in the supernatant of the cell suspension from swabs and brushes from each nostril, the pharynx, and trachea of both monkeys. Each supernatant was used to infect the virus-sensitive 293 cell line. Cytopathic changes in the 293 cells were monitored for 1 week and then the cells were fixed and stained for  $\beta$ -galactosidase activity. Cytopathic effects and blue-stained cells indicated the presence of live virus. In Monkey A, live virus was detected in both nostrils on day 3 after infection; no live virus was detected at either one or two weeks post-infection. In Monkey B, live virus was detected in both nostrils, pharynx, and trachea on day 3, and only in the infected nostril on day 7 after infection. No live virus was detected at 2 weeks or after.

*Stool culture.* Stools were collected every week and cultured for virus; all were negative.

*Adenovirus antibody titers.* Adenovirus antibody titers were measured by ELISA at the Iowa State Hygienic Laboratory. Adenovirus 2 antibody titers were measured by ELISA at Genzyme. Both monkeys had an increase in adenovirus 2 antibody titers.

## Conclusions

The results of these safety studies are reassuring in several respects. Neither monkey developed any signs associated with wild-type adenoviral infection. Although live virus was present in the nasal washings for at least 1 week (in one of the monkeys), there was no evidence of live virus at day 14 or beyond. Virus was present in the contralateral nostril and pharynx; we think the virus most likely reached those locations via drainage by gravity after we released the posterior occlusion or it may have been caused by the monkey putting its finger from one nostril to another after recovery from anesthesia. The monkeys received approximately 16-80 times the highest proposed human dose (on a PFU/cell basis), there were no adverse consequences and live virus rapidly disappeared. Finally, Ad2/BGal-1 was

not detected in stool samples taken for over one month, suggesting that ingestion of virus followed by replication in the gut does not occur.

**b. Ad2/CFTR-1 applied to the nasal epithelium.**

**Procedure**

The procedure for three monkeys (C, D, and E) was described above, Point B.2.b.(3). The monkeys were completely evaluated on days 1, 3, 6, 13, 20, and 27. The monkeys were also observed daily for any abnormal behavior or physical signs.

**Results**

*Examination.* The three monkeys tolerated the application procedure well. Visual inspection of the nasal epithelium revealed no inflammatory response. Daily examination revealed no evidence of coryza or conjunctivitis. There was no cough, sneezing, or diarrhea. Slight erythema was observed in all three monkeys in both the control (left) and virus (right) nostril on the first day after infection and then disappeared. Appetites and weights were not affected by virus administration. Monkey E had a poor appetite requiring special diet before the infection, but it improved throughout the experiment. Physical examination revealed no evidence of fever, lymphadenopathy, tachypnea or tachycardia at any time.

*Blood counts and serology.* One of the monkeys had a relative blood leukocytosis on day 6 post infection. The WBC increased to 8,000 from a preinfection count of 3,400. No abnormalities were found in the hematology of the other two monkeys. Serum ESR, electrolytes, transaminases, BUN and creatinine were normal throughout.

*Cytology and Histology of the nasal epithelium.* We assessed epithelial inflammation by cytological examination of Wright-stained cells obtained from brushings of the nasal mucosa. Wright stains of cells from the nasal mucosa were evaluated at each of the time points. Monkey C had a slight increase in inflammatory cells in both control and infected-nostrils on day 1; on subsequent days the number of inflammatory cells (lymphocytes, neutrophils, eosinophils) was less than 5%. Monkey D had less than 5% inflammatory cells throughout. Monkey E had an increased percentage of eosinophils in both nostrils at all the time points.

*Presence of virus on the airway epithelium.* We tested for the presence of virus in the supernatant of the cell suspension from swabs and brushes from each nostril and the pharynx. Cytopathic changes were monitored for 5 days and then the 293 cells were stained with an FITC-labeled anti-adenovirus monoclonal antibody (Chemicon). Live virus was detected on day 1 and 3, but not on day 7 or thereafter.

*Stool culture.* Stools were collected every week and cultured for virus; all were negative.

*Adenovirus antibody titers.* All three monkeys had an increase in adenovirus titers and adenovirus 2 antibody titers after infection.

**Conclusions**

None of the monkeys developed any signs associated with wild-type adenoviral infection. Significant eosinophilia was observed from nasal brushings of one of the monkeys; this was not associated with blood eosinophilia or with clinical signs of allergic rhinitis. As we indicate below, this was also observed in the bronchial brushings of some of the monkeys before virus administration and it was found in the sentinel monkey. Monkeys that had eosinophilia on any respiratory surface were monkeys E, F, G, and H. These four monkeys were recently transferred from San Francisco to Iowa; monkeys A and B have lived in Iowa

for several years; monkeys C and D were from Cleveland. We interpret the eosinophilia as an unrelated occurrence because: it was present in all of the San Francisco monkeys irrespective of the treatment group, it did not occur in the Iowa or Cleveland monkeys, and the eosinophilia preceded the virus administration. There were no other signs or manifestations of a significant inflammatory response. There was no evidence of viral replication. Finally, Ad2/CFTR-1 was not detected in stool samples suggesting that ingestion of virus followed by replication in the gut does not occur. We conclude that Ad2/CFTR-1 appears to be safe for topical application in the nasal mucosa.

**c. Ad2/BGal-1 applied to the bronchial epithelium.**

**Procedure**

The procedure for two monkeys (G and H) was described above, point B.2.b.(3). The monkeys were completely evaluated on days 1, 4, 7, 14, 21, 28, and 35. The monkeys were also observed daily for any abnormal behavior or physical signs.

**Results**

*Examination.* The two monkeys tolerated the infection procedure well. Neither monkey became cyanotic or bradycardic. We looked for a systemic inflammatory response by daily visual inspection of the nasal epithelium. Neither of the monkeys developed any evidence of coryza, conjunctivitis or diarrhea. There was no cough or sneezing. Physical examination revealed no evidence of lymphadenopathy, tachypnea or tachycardia. Monkey G had a rectal temperature 39.1° C on day 4 post infection and Monkey H had a rectal temperature of 39.4° C on day 1 and 39.2° C on day 4 post infection. Appetites and weights were not affected by virus administration in either monkey. Both monkeys underwent flexible fiberoptic bronchoscopy on each evaluation day. The bronchial mucosa from monkey H appeared normal throughout the experiment. Bronchial examination of monkey G revealed erythematous mucosa at the right main bronchus on days 1 and 4 post infection and appeared normal thereafter.

*Blood counts and serology.* Monkey H had a blood leukocytosis on day 4 post infection; the WBC increased to 11,300 with 8,380 neutrophils. No other abnormalities were found in the hematology, ESR, serum electrolytes, transaminases, BUN or creatinine.

*Cytology of the bronchial epithelium and bronchoalveolar lavage.* We assessed epithelial inflammation by cytological examination of Wright-stained cells obtained from brushings of the bronchial mucosa of the right bronchus intermedium, the left main bronchus and bronchoalveolar lavage. At baseline Monkey H had 7% eosinophils in the tracheal brushings. Both monkeys had a significant percentage of eosinophils in samples from right and left bronchial mucosa at all time points. Bronchoalveolar lavage from the right middle lobe revealed a significant number of eosinophils throughout the experiment in monkey G (10-46%) and on days 1, 4, and 7 in monkey H (29 - 39%). No significant lymphocytosis or increase in neutrophils was observed.

*Presence of virus replication on the airway epithelium.* At each of the time points we tested for the presence of live virus in the supernatant of the cell suspension from bronchial brushings and bronchoalveolar lavage as described above, using the virus-sensitive 293 cell line. In both monkeys, live virus was detected from the right bronchial brushings and bronchoalveolar lavage on day 1 post infection. On day 4 post infection live virus was detected in samples from bilateral bronchial brushings and bronchoalveolar lavage from monkey H. No live virus was detected from monkey G on day 4. There was no live virus from either monkey on day 7 or after.

*Stool culture.* Stools were collected every week and cultured for virus; all were negative.

*Adenovirus antibody titers.* Both monkeys had a significant rise in adenovirus 2 antibody titers.

### Conclusions

Both monkeys developed a transient low grade fever after infection with the recombinant virus. The procedures that these monkeys were exposed to may have contributed to the fever. About 10% of the human patients that undergo bronchoalveolar lavage develop fever within 24 hours. The bronchial instillation of a large number of viral particles may have been responsible for the fever and increase in WBC. Even though significant eosinophilia was observed in bronchial brushings and bronchoalveolar lavage, we do not believe that the eosinophilia is related to the virus for the reasons considered above in b. (Ad2/CFTR-1 applied to the nasal epithelium). Both monkeys had a significant rise in antibody titer to adenovirus. After a week we could no longer detect the presence of a live virus. Finally, Ad2/βGal-1 was not detected in stool samples suggesting that ingestion of virus followed by replication in the gut does not occur.

### d. Sentinel monkey.

#### Procedure

Monkey F was housed in the same room and was immediately adjacent to and surrounded by monkeys C, D, E, G, and H. Besides being used as sentinel, monkey F had exactly the same procedures as did monkey G and H, except that tris buffered saline was instilled in the right bronchus intermedius instead of Ad2/βGal-1. The monkey was completely evaluated on days 1, 4, 7, 14, 21, 28, and 35. The monkey was also observed daily for any abnormal behavior or physical signs.

#### Results

*Examination.* The monkey tolerated the mock infection procedure well, and did not become cyanotic or bradycardic. Visual inspection of the nasal epithelium revealed no inflammatory response. The monkey had no evidence of coryza, conjunctivitis or diarrhea. There were no changes in behavior. There was no cough or sneezing. Physical examination revealed no evidence of lymphadenopathy, fever, tachypnea or tachycardia. Appetite and weight were not affected. Flexible fiberoptic bronchoscopy revealed a normal appearance of the bronchial mucosa throughout the experiment.

#### *Blood counts and serology.*

Blood leukocytosis was seen on days 1 and 4 post infection, with neutrophil counts of 7,460 and 5,800/mm<sup>3</sup>, respectively. Serum electrolytes, transaminases, BUN, ESR, and creatinine were normal throughout.

*Cytology of the bronchial epithelium and bronchoalveolar lavage.* We assessed epithelial inflammation by cytological examination of Wright-stained cells obtained from brushings of the bronchial mucosa of the right bronchus intermedius and bronchoalveolar lavage. A significant percentage of eosinophils in samples from right bronchial mucosa was noted on days 1, 4, post instillation of placebo. Bronchoalveolar lavage from the right middle lobe revealed significant number of eosinophils throughout the experiment (12-52%). No significant lymphocytosis or increase in neutrophils was observed.

*Presence of virus on the airway epithelium.* At each of the time points, we tested for the presence of virus in the supernatant of the cell suspension from bronchial brushings and bronchoalveolar lavage. No virus was detected at any point of the experiment.

*Stool culture.* Stools were collected every week and cultured for virus; all were negative.

*Adenovirus antibody titers.* No increase in antibody to adenovirus or adenovirus 2 was detected at any of the time points.

**Conclusion.**

The results of this experiment are important in several respects. Despite being housed in close proximity to two monkeys treated with Ad2/βGal-1 bronchial instillation and 3 monkeys treated with Ad2/CFTR-1 virus, the sentinel monkey developed no increase in antibody titers to the virus. Moreover, no live virus was isolated at any point from the lung or nose or from stool samples of the sentinel monkey. The fact that the sentinel monkey had eosinophilia on bronchial brushings and bronchoalveolar lavage, suggests that it was not due to the recombinant virus.

**B.3. Clinical procedures, including patient monitoring.**

**Describe the treatment that will be administered to patients and the diagnostic methods that will be used to monitor the success or failure of the treatment. If previous clinical studies using similar methods have been performed by yourself or others, indicate their relevance to the proposed study.**

**1. Study design.**

This is a nonrandomized, nonblinded administration of recombinant Ad2/CFTR-1 virus to a defined and limited area of respiratory nasal epithelium.

**2. Pretreatment evaluation.**

To insure clinical stability, participants will enter the study at least 6 weeks before administration of virus. They will then be followed as outpatients at 3 to 4 week intervals until admission for application of the recombinant virus. Participants will receive the evaluation described below at the time of entry into the study and then at the time of admission to the hospital. They may also receive any of the following tests between enrollment and administration of the recombinant virus at the discretion of the investigators, if there is a significant change in clinical status.

a. Complete history and chart review and assessment by NIH scoring system.

b. Physical examination, including nasal endoscopy.

c. CF genotyping.

d. Blood samples for: complete blood count (including platelets and differential WBC), erythrocyte sedimentation rate, electrolytes (including Na, K, Cl, and HCO<sub>3</sub>), and general chemistries (including total protein, albumin, Ca, PO<sub>4</sub>, glucose, uric acid, alkaline phosphatase, total bilirubin, AST, LDH, BUN, Cr, and amylase).

e. Adenovirus antibody titers.

f. Cultures. Culture of sputum, nasal swab, stool, and urine for wild-type adenovirus. Bacterial culture of sputum and nasal swab.

g. Nasal swab and nasal brushing for cell count and cell morphology and PCR for E1 DNA sequences.

- h. Measurement of transepithelial electrical potential difference across the nasal epithelium.
- i. Pulmonary function tests including spirometry, lung volumes, and diffusing capacity.
- j. Arterial blood gases.
- k. Chest X-ray: P-A and lateral.
- l. Computerized tomography of the sinuses (at enrollment into the study and at the completion of the study only, unless indicated by a change in clinical status).

### 3. Virus Application.

Patients will be admitted to the Clinical Research Center at the University of Iowa on the day of virus administration. They will be placed in isolation in a hospital room with complete isolation precautions (see Appendix 4).

Endoscopic examination of the nasal mucosa will be used to identify the optimal treatment area and to define well recognized landmarks. We will use the area along the inferior surface of the inferior turbinate. The area will be prepared by gentle rinsing with saline and suction.

To minimize sneezing, discomfort, and movement during application of virus, patients will receive 2% topical lidocaine to the nasal mucosa immediately before application. As mild sedation and to prevent movement during the application, they will also receive Midazolam 1-2 mg. IM. shortly before application.

The recombinant virus will be applied to a defined area of nasal mucosa using an application area of approximately  $0.5 \text{ cm}^2$  in each nares. We will use a plexiglass applicator that allows us to apply the virus to a defined area and then wash it away; in preliminary experiments we have tested the applicator in normal humans using methylene blue as a tracer. The virus will remain in contact with the nasal epithelium for 30 min. Then the mucosa will be gently washed to remove unattached virus.

Different doses of the virus/gene construct will be used.

<u>PATIENT</u>	<u>TOTAL DOSE*</u>	
	PFU	$\mu\text{g}$
1	$2 \times 10^6$ PFU	0.25
2	$2 \times 10^7$ PFU	2.5
3	$5 \times 10^7$ PFU	6.25

\*Preparation and assay of production lots of virus is described under B.1.b.(1)(b). We will apply the virus in a volume of 50 to 200  $\mu\text{l}$  to an area of approximately  $1 \text{ cm}^2$  of nasal mucosa. Assuming that  $1 \text{ cm}^2$  of nasal mucosa has about  $2 \times 10^6$  cells, a dose of  $2 \times 10^6$  PFU corresponds to a dose of approximately 1 PFU/cell. Based on our experiments with human airway epithelia, we expect that 10 PFU/cell will provide substantial correction of the CF

chloride transport defect. For comparison, in a recent human trial using an E3 replacement adenovirus vector,  $1.6 \times 10^7$  PFU were administered (93).

#### **4. Evaluation after virus application.**

After application of the virus, we estimate that the patient will be evaluated for approximately 14-16 days in the hospital. During the hospitalization, the patient will be confined to the hospital room. If it is required that they leave the room for specific procedures, they will wear a mask to cover the nose and mouth during such times out of the room. Strict respiratory, enteric, and body fluid isolation will be used. The patient will not be discharged from the hospital until two consecutive cultures for live virus from nasal swabs are negative. (Note that 7-8 days may be required to determine that a culture is negative.) Thus, the duration of hospitalization could potentially be longer than two weeks.

During hospitalization, the patient's standard therapies for CF will continue. To maximize safety, results from each patient will be evaluated before proceeding to the next patient.

Specific assessments performed on each patient will include the following (the timing of specific assessments is given in the protocol, Appendix 1):

##### **a. History and physical examination.**

*Purpose:* To obtain evidence of patient discomfort, systemic responses, or inflammation.

*Methods:* The patient will be questioned about local or systemic manifestations of inflammation. Vital signs will be recorded. The area of application of the recombinant virus will be visually examined by endoscopy and compared to nontreated epithelium. We will look for evidence of exudate, inflammation, edema, or erythema.

##### **b. Blood/serum by venipuncture.**

*Purpose:* To assess the systemic response to virus application and the antibody response to recombinant virus.

*Methods:* Venous blood will be collected by standard venipuncture technique. Analysis will include the blood count and chemistry evaluations described above in the pretreatment evaluation. Antibody titers to the recombinant Ad2/CFTR-1 adenovirus will be determined by ELISA.

##### **c. Viral cultures of nasal and pharyngeal swabs, nasal brushing, blood buffy coat, urine, and stools.**

*Purpose:* To determine the presence of recombinant virus.

*Methods:* All samples will be cultured on virus permissive 293 cells. We expect live virus to be present in the initial swabs but then to disappear with time. If there is viral replication, we expect that after an initial decline, the titer of live virus in the nasal swab would increase. We will determine whether wild-type or recombinant virus is produced by restriction enzyme analysis.

**d. Brushing and swab of nasal and pharyngeal mucosa for evaluation of an inflammatory response.**

*Purpose:* To determine the presence of an inflammatory response.

*Methods:* For brushing, the nasal mucosa will be treated with 5 drops of Afrin (oxymetazoline hydrochloride 0.05% solution, Schering-Plough) and up to 1 ml of 2% Lidocaine five min before brushing. A cytobrush (used for PAP smears) will be used to gently brush the nasal mucosa for approximately 3 sec at the site of administration of the recombinant virus. The brush will then be removed and the cells dissociated from the brush into 2 ml of phosphate buffered saline. The cell suspension will be kept on ice until further use. The nasal mucosa from each nostril and the pharyngeal mucosa will be swabbed with a cotton-tipped applicator.

Evidence of an inflammatory response will be assessed by cytological examination of cytospin preparations of the cells using Wright stain. The cell differential count will be determined for each specimen. Cell morphology and cytopathic effects will be evaluated using the PAP stain.

**e. Biopsy of nasal epithelium.**

*Purpose:* The nasal epithelium will be biopsied for evaluation of an inflammatory infiltrate into and beneath the epithelium and for evaluation of cytopathic effects.

*Methods:* The patient's nose will be anesthetized using, initially, a solution of eight parts 1% Pontocaine and 2 parts 1/1,000 epinephrine which will be topically applied using neurosurgical cottonoid pledgets. The pledgets will be placed under direct vision utilizing endoscopic control. After ten minutes the area of the biopsy will be submucosally infiltrated with 0.5 ml of 1% xylocaine with 1/100,000 epinephrine, to ensure anesthesia and aid hemostasis. Approximately 0.5 cm<sup>2</sup> of nasal mucosa will be resected from the area of virus application under telescopic control. This area will be identified by its relation to certain fixed landmarks in the nose, e.g. anterior end of inferior turbinate, cartilaginous nasal septum, membranous nasal septum. Immediately after the biopsy has been taken, a further cottonoid soaked in oxymetazoline hydrochloride 0.05% solution, will be placed on the biopsied area to effect hemostasis. We do not anticipate having to use nasal packing. The patient will be treated postoperatively with Tylenol as required for analgesia.

The biopsy will be evaluated for the integrity of the epithelium and for inflammatory changes by conventional light microscopy, including hematoxylin and eosin stain. Slides will be reviewed by an independent pathologist. Electron microscopy will also be performed on the section.

**f. Nasal brushing and biopsy for evaluation of vector efficacy.**

*Purpose:* To evaluate the ability of Ad2/CFTR-1 to produce CFTR mRNA and protein.

*Methods:* In cells from the nasal brushings, RT-PCR will be used to assess the presence of virally-produced mRNA. Evidence for mRNA production and its location will also be obtained by *in situ* hybridization.

Evidence of CFTR protein production and location at the apical membrane will be assessed by immunocytochemistry using monoclonal antibodies directed against CFTR: M1-4 and

M13-1. These studies will be performed on the cytospin preparations from nasal brushing and on the biopsy specimen.

**g. Transepithelial electrical potential difference across the nasal epithelium.**

*Purpose:* To assess the electrophysiological consequences of treatment. Patients with CF have an abnormally increased transepithelial electrical potential difference across the nasal epithelium (103,104). In addition, the response of the electrical potential difference to several agents that regulate transport is abnormal. The abnormalities in patients with CF are well characterized and relatively easy to measure. Correction of the abnormality would indicate that administration of the recombinant virus has corrected the CF electrolyte transport defect in a localized area of nasal epithelium.

*Methods:* Measurement of the nasal PD is safe, easy, and not invasive (103,104). The reference bridge consists of a polyethylene PE 240 tubing filled with 3 M KCl with 4% agar placed under an EKG patch (3-M) on the forearm. The reference bridge is connected through a calomel cell to a voltmeter (602 electrometer, Keithly Instruments). The exploring bridge is a small PE 50 tubing perfused with saline solution (0.2 ml/min, Harvard Compact Infusion Pump) which is connected to a 3 M KCl bridge and then a calomel cell. The electrical potential difference between the exploring and reference electrodes is measured by a voltmeter. Output of the voltmeter is connected to a strip chart recording (Servocorder SR 6253 Datamark). The exploring electrode (the PE50 tubing) will be used to gently touch the site of virus application under direct vision. After a five minute baseline, the infusion solution will be changed to one consisting of Ringer's solution containing either 100  $\mu$ M terbutaline, 100  $\mu$ M amiloride, 100  $\mu$ M adenosine, and/or saline in which the chloride concentration has been reduced by replacement with gluconate. The entire procedure will take between 15-30 minutes. Treated and non-treated adjacent mucosa will be analyzed for each condition.

**h. Mucosal fluid for adenovirus antibody.**

*Purpose:* To test for the presence on the mucosal surface of IgA or IgG antibody to the recombinant adenoviruses and to test for neutralizing antibodies.

*Methods:* Before administration of the virus and at one month after we will obtain a washing of the nasal surface to test for the presence of specific and neutralizing antibody.

**5. Evaluation after discharge.**

After discharge from the Clinical Research Center, patients will be evaluated at weekly or every other week intervals for 6 weeks. We will continue to make the same assessments as described above. The data may be useful in assessing the persistence of expression of CFTR.

**B.3.a. Will cells (eg, bone marrow cells) be removed from patients and treated ex vivo? If so, what kinds of cells will be removed from the patients, how many, how often, and at what intervals?**

No.

**B.3.b. Will patients be treated to eliminate or reduce the number of cells containing malfunctioning genes (eg, through radiation or chemotherapy)?**

No.

**B.3.c. What treated cells (or vector/DNA combination) will be given to patients? How will the treated cells be administered? What volume of cells will be used? Will there be single or multiple treatments? If so, over what period of time?**

The Ad2/CFTR-1 gene construct will be prepared at Genzyme, as described above. Appropriate aliquots of purified production lots of virus will be diluted to 50 - 200  $\mu$ l in Tris buffered saline. There will be a single, 30 min. application of virus to each nares. The virus will be applied to each nares using a plexiglass applicator that will limit the area of application to a defined 0.5 cm<sup>2</sup> area and will allow the majority of virus to be removed and the area to be rinsed with virus-free solution after application.

**B.3.d. How will it be determined that new gene sequences have been inserted into the patient's cells and if these sequences are being expressed? Are these cells limited to the intended target cell populations? How sensitive are these analyses?**

As described above in response to point B.3., we will assess the production of mRNA by RT-PCR and *in situ* hybridization, we will assess the production of protein by immunocytochemistry, and we will assess the correction of the CF electrolyte transport defect by measurement of the transepithelial electrical potential difference across the nasal epithelium. We will examine the treated areas and those immediately adjacent.

**B.3.e. What studies will be done to assess the presence and effects of the contaminants?**

The Ad2/CFTR-1 will be purified and tested for the presence of biological and protein contaminants. The most likely contaminant in the preparation is BSA from the serum used to grow the virus. If detected in the vector preparation, we will examine patient serum for the presence of antibodies to BSA in a Patient Immune Response (PIR) assay. Genzyme routinely develops similar assays to measure antibody responses to other therapeutic proteins. However since it is likely that patients will already have antibodies to BSA this test is likely to be of limited value.

**B.3.f. What are the clinical endpoints of the study? Are there objective and quantitative measurements to assess the natural history of the disease? Will such measurements be used in following patients? How will patients be monitored to assess specific effects of the treatment on the disease? What is the sensitivity of the analyses? How frequently will follow-up studies be done? How long will patient follow-up continue?**

The biochemical endpoints and methods for assessment are described above in response to Point B.3. Because virus administration is limited to a small area of epithelium in the nose, we expect no effect on the clinical disease. Nevertheless, we will assess the patient's clinical status as described in Point B.3.

**B.3.g. What are the major beneficial and adverse effects of treatment that you anticipate? What measures will be taken in an attempt to control or reverse these adverse effects if they occur? Compare the probability and magnitude of potential adverse effects on patients with the probability and magnitude of deleterious consequences from the disease if recombinant DNA transfer is not used.**

The major beneficial effect of this study will be the acquisition of knowledge that will allow us and others to progress in the treatment of this disease. While it is unlikely that the present protocol will be of benefit to the participants, future protocols or treatments that may emerge as a result of this work could be of significant benefit to patients with CF.

Potential risks and adverse effects of the protocol include the following.

a. Risks associated with inflammation, cytopathic effects, and immune response.

It is possible that inflammation locally at the site of application of the virus could occur. Local cytopathic effects on the respiratory nasal epithelium are also possible. Inflammation or cytopathic effects include local pain, bleeding or subsequent scarring; although serious effects appear very unlikely. The risk is minimized by the use of small doses of the virus in a very limited area of nasal mucosa. The use of nasal mucosa, rather than bronchial mucosa, is a major safety feature that should allow us to assess safety without endangering the patient. Our animal studies, which used much higher doses of virus, indicate that if inflammation should occur, it will be mild and transient. Our studies with human airway epithelia suggest that there will be no cytopathic effects.

It is possible that the administration of Ad2/CFTR-1 could produce an immune response to the recombinant virus that was not present before the study. While it is unlikely that this would affect the patient during the study, the presence of immune sensitization could affect the response to subsequent treatment with adenovirus vectors. However, the risk to the participants of this study will be minimized because they will already be seropositive. These considerations may be more of an issue in seronegative patients or with repeated administration of the virus.

b. Risks associated with virus replication.

The Ad2/CFTR-1 viral construct has been rendered defective for replication by deletion of two important early genes E1a and E1b. However, it is possible that the virus will have a limited ability to replicate in human cells. Furthermore, under certain circumstances, the viral defect could be complemented. Such circumstances include low level contamination of the Ad2/CFTR-1 preparation with wild-type virus, coinfection with wild-type adenovirus, or provision of E1 gene function by latent or residual adenovirus resulting from an earlier infection (100), or by infection by other viruses able to provide such function (127-129). Finally, epithelia might also provide normal cellular proteins with E1-like functions that are able to complement the defective virus (101,102).

Most of these possibilities seem remote. The Ad2/CFTR-1 virus is defective not only because it lacks E1, but also because it is so large as to be difficult to package. Thus, even in permissive cells that provide E1 functions, virus replication is modest relative to wild-type. Second, the likelihood of coinfection of treated cells with wild-type virus is minimized by prior screening of the patients, by treating only seropositive patients, by treating only a small area of nasal epithelia and by isolation of the patient during treatment. Third, coinfection with another virus, although possibly providing some E1 activities, is unlikely to complement all E1 gene functions (145). Fourth, in the unlikely event that coinfection or contamination with wild-type adenovirus did occur, the wild-type virus would probably compete out the defective virus resulting, eventually, in a wild-type infection that would be expected to become self-limited. Model experiments, B2a(4), confirm that wild-type virus rapidly overgrows Ad2/CFTR-1. Because there is data to suggest the presence of E1 sequences from previous adenovirus infections in some individuals (100), we will screen patient's nasal cells for E1 DNA by PCR and exclude those patients with detectable signal.

There is also a possibility of recombination between wild-type virus and the Ad2/CFTR-1 virus. Whether this occurred by legitimate or illegitimate recombination, for any progeny virus to be viable would require two events to occur. First, insertion of the missing E1

gene and second, deletion of DNA sequences to enable the resulting recombinant to become small enough to package its DNA. The most likely sequences to be deleted, compatible with retention of viral replication, would be some or all of the CFTR DNA. Growth of such a recombinant would probably be self-limiting and would be unlikely to lead to synthesis of CFTR fragments with any significant biological activity.

Our animal and cultured cell studies, some using doses greatly in excess of the human dose, have revealed no evidence for Ad2/CFTR-1 replication. Moreover, we found no evidence for Ad2/βGal-1 replication despite its smaller size and more ready growth in 293 cells.

c. Effects of wild-type adenovirus 2 infection.

Potential effects of wild-type Ad2 include symptoms of upper respiratory infection. Pharyngitis and conjunctivitis are known to be associated with adenovirus. Strains of adenovirus other than Ad2 have been known to cause pneumonia, transient diarrhea and gastroenteritis. Rare problems associated with adenoviral strains include cystitis and keratoconjunctivitis. Such problems could be hazardous in immunocompromised hosts. Risk is minimized in the protocol by the use of patients who have only mild to moderate disease and who have evidence of preexisting immunity to adenovirus. The possible role of adenovirus in oncogenesis is discussed in Section 4.4; there is no evidence to suggest a role for adenovirus in human tumors.

d. Risks associated with the study procedures.

i. Measurement of the transepithelial electrical potential difference across the nasal epithelium will not produce significant discomfort; it has generally been well tolerated in the past. The drugs applied topically to the nasal epithelium during the course of the study have no significant local or systemic effects.

ii. Nasal and pharyngeal swabs can produce mild discomfort when they are taken, but there is no serious or long term risk. They are standard practices in clinical outpatient medicine.

iii. Brushing the nasal mucosa could produce discomfort that might persist for several hours. It could also produce some minor bleeding (a few ml at most). There are no major or long term risks.

iv. The process of application of the recombinant virus to the nasal mucosa should have no significant risk. There could be minor discomfort or a sense of nasal obstruction. There may also be discomfort from having to remain still during the procedure. These discomforts should be minimized by local anesthesia with topical 2% lidocaine to the nasal mucosa and by administration of Midazolam 1-2 mg. IM. shortly before application. The major risks of lidocaine and Midazolam would be hypersensitivity which is very rare. The Midazolam could theoretically produce some respiratory depression; however the dose we will use should have no significant effects, particularly in these subjects who will only have mild to moderately severe disease.

v. Biopsy of the nasal epithelium will produce mild to moderate pain and discomfort. Pain and discomfort during the procedure will be minimized by use of local anesthesia. Subsequent use of analgesics will be allowed if needed. A small risk of bleeding or subsequent scar formation after the biopsy is possible as is expected for such a routine procedure. There is also a risk of infection. We will treat pain with analgesics and observe the area for bleeding or infection; if either should require it, we will administer appropriate treatment.

- vi. Venipuncture to obtain the blood samples produces minimal risks that are well-known.
- vii. Arterial puncture for measurement of blood gases includes the risk of bleeding and bruise formation and pain at the time of the procedure. The procedure is standard and done frequently on outpatients.
- viii. Pulmonary function studies are routine on outpatients with a variety of lung diseases. There are no anticipated short or long term risks.
- ix. Chest and sinus X-rays carry the risk of radiation exposure; we anticipate that a subject will have three chest X-rays and two computerized tomographic X-rays of the sinuses during the course of the studies. Although there are no known adverse effects of this amount of radiation, the long term effects of such radiation are not known with certainty.
- x. Emotional discomfort related to respiratory isolation in a hospital room for two weeks may occur. We will provide reading material, VCRs, an exercise bicycle, etc. to the patient. If required we will provide psychological support.

**B.3.h. If a treated patient dies, what special post mortem studies will be performed?**

It is not anticipated that death should occur during participation in the trial. Should it occur for any unforeseen reason, we will request an autopsy. This is discussed in the Information Summary for Informed Consent.

**B.4. Public Health Considerations**

**Describe any potential benefits and hazards of the proposed therapy to persons other than the patients being treated. Specifically:**

**B.4.a. On what basis are potential public health benefits or hazards postulated?**

The knowledge obtained from this study could lead to a significant improvement in the care of patients with CF. Thus, because CF is a common, chronic, debilitating disease which is usually lethal, it could have a significant beneficial public health impact.

There is a risk that the virus could be passed to another person. This could occur if a caregiver or visitor is exposed to the virus we apply to the patient or exposed to virus that had replicated in the patient. Wild-type adenovirus 2 causes mild disease. Because Ad2/CFTR-1 is replication impaired and because our experimental results have demonstrated no virus replication, the possibility that an exposed person will develop clinical disease is very remote.

Were environmental release to occur, the most likely virus involved would be wild-type adenovirus that had overgrown the Ad2/CFTR-1 or a recombinant with no biological activity other than that associated with wild-type virus. Recombinant and wild-type adenovirus has been used previously in human populations (93,146).

**B.4.b. Is there a significant possibility that the added DNA will spread from the patient to other persons or to the environment?**

Both theoretical considerations and experimental results suggest that the risk of spread to other persons or to the environment is very low. a) Our data indicate that viral replication in the patient is very unlikely. Thus, given the low amounts of virus used, it is unlikely that the virus will spread. b) As indicated below, we will take precautions to decrease the risk

of spread to another person. c) Our data suggest that the sentinel monkey was not exposed to the adenovirus. d) Studies using live wild-type adenovirus vaccines show little horizontal transmission between roommates (146).

**B.4.c. What precautions will be taken against such spread (e.g., to patients sharing a room, health-care workers or family members)?**

We will take several precautions to minimize the possibility of spread.

The amount of virus that will be applied is very small; thus the possibility of transfer is minimized.

Hospital care will be administered by trained health-care workers. The nurses and care givers in the University of Iowa Clinical Research Center are skilled at precisely following experimental protocols. In addition, we have sought the advice of Dr. Richard Wenzel, M.D., Hospital Epidemiology, and Ms. Marlene Schmid, Senior Nurse Epidemiologist, in minimizing the possibility of spread of the virus. We will follow their recommendations (Appendix 4). Healthcare workers will be informed of the nature of the study and of the potential risk to immunocompromised persons they may also care for.

To minimize the risk of exposure to persons other than the participant, the patient will be kept in isolation in a hospital room in the Clinical Research Center. Persons coming in contact with the participant will practice hand washing and use gowns, gloves, and masks, which will be removed upon leaving the room.

In addition to the strict control placed on the exposure of the patient to his/her surroundings, as previously discussed, Ad2/CFTR-1 is defective and because of its large size, difficult to package. Thus, its ability to replicate on its own is doubly compromised.

The participant will not be discharged from the isolation room until there are two consecutive nasal swabs that are negative for live virus. If the nasal swabs and brushes should remain positive for recombinant adenovirus for 4 weeks after infection and if the patient is showing no adverse clinical effects, we will discharge them from the hospital at that point. We will, however, continue to evaluate them at weekly intervals. We believe that to continue to confine the patient to the room at that point would be inhumane. Moreover, if no adverse effects have been observed, the risk to the general public and the environment should be minimal. Most importantly in this regard, our safety studies in cultured cells and in animals and reports from the literature suggest that the possibility that the patient will continue to shed virus or pass it to another person is remote (146).

**B.4.d. What measures will be undertaken to mitigate the risks, if any, to public health?**

The measures are described above in section B.4.c.

**B.4.e. In light of possible risks to offspring, including vertical transmission, will birth control measures be recommended to the patient? Are such concerns applicable to health care personnel?**

As described in the inclusion and exclusion criteria for patient selection, all participants must practice contraception for at least one month before and after the protocol. Greater than 97% of men with CF are sterile. Women participants must have a negative pregnancy test. Given the exceedingly low theoretical risk, the results of our animal experiments

testing for transfer to other tissues including the gonads in hamsters, and the precautions we take, we believe the risk to offspring of health care personnel is exceedingly low.

**B.5. Qualifications of investigators, adequacy of laboratory and clinical facilities.**

**Indicate the relevant training and experience of the personnel who will be involved in the preclinical studies and clinical administration of recombinant DNA. In addition, please describe the laboratory and clinical facilities where the proposed study will be performed.**

**B.5.a. What professional personnel (medical and nonmedical) will be involved in the proposed study and what is their relevant expertise? Please provide curricula vitae of key professional personnel ( see Section III-E).**

Drs. Michael J. Welsh and Alan E. Smith and their collaborators have published extensively on CF and CFTR (1,7,42,62,147,148). Their work on CFTR showed that expression of CFTR complemented the CF defect (9,22), elucidated the function of CFTR (24,33-35,45-47,149), and explained how mutations in CFTR cause a loss of function (32,48,57,58,60).

Dr. Alan E. Smith is Senior Vice President - Research at Genzyme Corporation, and is widely experienced in molecular biology and animal virology. Dr. Smith has published extensively on picornaviruses, arboviruses, retroviruses and papovaviruses and from 1977-1981 was on the Editorial Board of Journal of Virology. Genzyme manufactures and sells Ceredase, a drug used to treat Gauchers Disease. Genzyme has also developed the recombinant proteins, thyroid stimulating hormone and glucocerebrosidase, that are presently in clinical trials. Genzyme will produce and test the Ad2/CFTR-1 vector.

Procedures involving the patient will be performed by Drs Michael J. Welsh, M.D., Joseph Zabner, M.D., and Scott M. Graham, M.D. Each has substantial clinical experience, including the care of patients with communicable, respiratory diseases. In addition, Dr. Scott M. Graham, currently performs the majority of the sinus surgery at the University of Iowa and is highly skilled in the diagnosis and treatment of disorders that affect the nasal mucosa. For the last three years, Dr. Graham has been course director for an internationally attended course in Advanced Techniques in Endoscopic Sinus Surgery. The nurses and caregivers in the Clinical Research Center are highly skilled at accomplishing complex experimental protocols and have experience in caring for patients in isolation. Copies of the investigator's curricula vitae are located in Appendix 7.

**B.5.b. At what hospital or clinic will the treatment be given? Which facilities of the hospital or clinic will be especially important for the proposed study? Will patients occupy regular hospital beds or clinical research center beds? Where will patients reside during the follow-up period? What special arrangements will be made for the comfort and consideration of the patients? Will the research institution designate an ombudsman, patient care representative, or other individual to help protect the rights and welfare of the patient?**

The study will be performed at the University of Iowa Medical Center in the Clinical Research Center. The University of Iowa Medical Center has all of the facilities required for the care of the patients; it is the largest teaching hospital in the country. Patients will be in single rooms in isolation. After there is no further evidence of live virus by two consecutive cultures, the patients will be discharged to their homes. We will try to offer entertainment (VCR, reading material, games), a stationary bicycle, and psychological

assistance, if needed. On the Certification of Subject Consent, we identify a person that the patient may contact for complaints and for help in protecting their rights and welfare.

### **C. SELECTION OF PATIENTS.**

**Estimate the number of patients to be involved in the proposed study. Describe recruitment procedures and patients eligibility requirements, paying particular attention to whether these procedures and requirements are fair and equitable.**

#### **C.1. How many patients do you plan to involve in the proposed study?**

Three.

#### **C.2. How many eligible patients do you anticipate being able to identify each year?**

All three patients will be identified in the first year.

#### **C.3. What recruitment procedures do you plan to use?**

The patients will be referred to the study for consideration by his/her physician. We anticipate, but do not require, that most participants will be patients at the University of Iowa Hospitals and Clinics.

#### **C.4. What selection criteria do you plan to employ? What are the exclusion and inclusion criteria for the study?**

##### **Inclusion Criteria**

- a. Patients with CF with mild to moderate severity of disease. Patients should score above 70 using the CF NIH scoring system (150).
- b. Male or female patients, age greater than 18 years.
- c. CF genotype:  $\Delta F508$  homozygous is preferred. If patients homozygous for  $\Delta F508$  are not available, we will use patients who are  $\Delta F508$  compound heterozygotes. In the case of  $\Delta F508$  compound heterozygotes, the mutation on the other chromosome must be known to cause CF and must not be associated with a milder clinical phenotype.
- d. Seropositive for antibody to adenovirus. Seropositivity is very common (greater than 70%) in the general population (76). Seropositivity is required to insure a rapid and adequate immunologic response to the virus and to minimize any potential for dissemination of the virus.

##### **Exclusion Criteria**

- a. Current instability of respiratory status.
- b. Hypoxemia with  $P_aO_2$  less than 66 mm Hg.
- c.  $FEV_1$  less than 50 % of predicted.

- d. Weight for height (%) less than 90% of predicted.
- e. Pregnancy: all subjects must practice contraception for at least one month and will have a negative test for pregnancy.
- f. Patient has school age children.
- g. Chronic severe nasal/sinus disease, either infectious or allergic in nature. Persistent purulent nasal discharge, obstructing nasal polyps, significantly inflamed nasal mucosa, or symptomatic sinusitis requiring repeated antibiotic therapy will exclude patients from the study.
- h. Upper respiratory infection of patient or household member within two weeks prior to entry.
- i. Corticosteroid therapy within the last 4 months.
- j. Current use of inhaled DNase therapy.
- k. Chronic adenoviral shedding within six weeks of study as detected in blood, urine, and nasal swabs.
- l. Patients may be excluded after the initial evaluation if PCR of the cells brushed from the nasal epithelium shows evidence of adenovirus 2 or 5 E1 genes (detected by PCR as described in Point B.1.b(1)(b) 8).
- m. Subjects may not participate in other research protocols during the proposed study.
- n. Inability to comprehend nature of the study or to give informed consent.

**C.5. How will patients be selected if it is not possible to include all who desire to participate?**

Those first three patients judged most able to participate in the investigation will be chosen.

**D. INFORMED CONSENT**

Indicate how patients will be informed about the proposed study and how their consent will be solicited. The consent procedure should adhere to the requirements of DHHS regulations for the protection of human subjects (45 Code of Federal Regulations, Part 46). If the study involves pediatric or mentally handicapped patients, describe procedures for seeking the permission of parents or guardians and, where applicable, the assent of each patient. Areas of special concern highlighted below include potential adverse effects, financial costs, privacy, long-term follow-up and post mortem examination.

**D.1. How will the major points covered in Sections I-A through I-C of this document be disclosed to potential participants in this study and/or parents or guardians in language that is understandable to them?**

In accordance with 21 CFR Part 46, informed consent to participate in this clinical study will be obtained from the patient by the investigator prior to initiating treatment. The patient will receive a copy of the Information Summary to read and discuss with the

investigator. This consent form was prepared in language that is easy to read and understand. The patient has the right to ask questions at any time. The study does not involve minors or handicapped individuals. The consent form is attached.

- D.2. How will the innovative character and the theoretically possible adverse effects of the experiment be discussed with patients and/or parents or guardians? How will the potential adverse effects be compared with the consequences of the disease?**

See the Consent Form (Appendix 2).

- D.3. What explanation of the financial costs of the experiment, follow-up care, and any available alternatives will be provided to patients and/or parents or guardians?**

The attached Consent Form contains this information. Patients will not be responsible for the costs of this study. Patients will be compensated for the time and inconvenience involved in participating in the research in the amount of \$75 for each day that they spend in the hospital. In addition, the patient will be reimbursed for travel expenses to and from the hospital for each day the patient comes to the clinic for an outpatient visit. The rate of reimbursement will be \$0.25 per mile and will include parking expenses.

- D.4. How will patients and/or their parents or guardians be informed that the innovative character of the experiment may lead to great interest by the media in the research and in treated patients?**

The investigator will discuss this with the patients.

- D.5. How will patients and/or their parents or guardians be informed:**

- D.5.a. About the irreversible consequences of some of the procedures performed?**

See attached Consent Form (Appendix 2).

- D.5.b. About any adverse medical consequences that may occur if a subject withdraws from the study once it has begun?**

See attached Consent Form (Appendix 2).

- D.5.c. About a willingness to cooperate in long-term follow-up will be expected?**

- D.5.d. About expectations that permission to perform an autopsy will be granted in the event of a patient's death following transfer as a precondition for a patient's participation in the study? This stipulation is included because an accurate determination of the precise cause of a patient's death would be of vital importance to all future patients.**

This expectation is contained in the Information Summary For Informed Consent (Appendix 2).

## **E. PRIVACY AND CONFIDENTIALITY**

Indicate what measures will be taken to protect the privacy of patients and their families as well as to maintain the confidentiality of research data.

### **E.1. What provisions will be made to honor the wishes of individual patients (and the parents or guardians of pediatric or mentally handicapped patients) as to whether, when, or how the identity of patients is publicly disclosed?**

The U.S. Food and Drug Administration (FDA) and Genzyme Corporation may inspect and copy medical records relating to this study, and the results of the study will be reported to the FDA and perhaps to other regulatory agencies. This information will be treated confidentially and, in the event of any publication regarding this study, patient identity will not be disclosed.

### **E.2. What provision will be made to maintain the confidentiality of research data, at least in cases where data could be linked to individual patients?**

A record of patient participation in this research will be maintained. This record will be kept confidential. The patient will be assigned a study number and the information will be kept under this study number rather than the patient name.

## **II. SPECIAL ISSUES**

Although the following issues are beyond the normal purview of local IRBs, the RAC and its Subcommittee request that investigators respond to questions A and B below.

### **A. What steps will be taken, consistent with point I-E above, to ensure that accurate and appropriate information is made available to the public with respect to such public concerns as may arise from the proposed study?**

We will coordinate a response to inquiries from the press with the University of Iowa Health Sciences Affairs Office, the Howard Hughes Medical Institute, and Genzyme Corporation.

### **B. Do you or your funding sources intend to protect under patent or trade secret laws either the products or the procedures developed in the proposed study? If so, what steps will be taken to permit as full communication as possible among investigators and clinicians concerning research methods and results?**

Genzyme Corporation will protect the product and procedure developed in the study. We plan to publish all findings from the study.

## **III. REQUESTED DOCUMENTATION**

In addition to responses to the questions raised in these Points to Consider, please submit the following materials:

### **A. Your protocol as approved by your local IRB and IBC.**

Protocol: Appendix 1. Informed Consent: appendix 2

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**B. Results of local IRB and IBC deliberations and recommendations that pertain to your protocol.**

Appendix 3

**C. A one-page scientific abstract of the protocol.**

Appendix 4

**D. A one-page description of the proposed experiment in nontechnical language.**

Appendix 5

**E. Curricula vitae for key professional personnel.**

Appendix 6

**F. An indication of other federal agencies to which the protocol is being submitted for review.**

The protocol will be submitted to the Food and Drug Administration.

**G. Any other material which you believe will aid in the review.**

**IV. REPORTING REQUIREMENTS**

**A. Serious adverse effects of treatment should be reported immediately to both the local IRB and the NIH Office for Protection from Research Risks and a written report should be filed with both groups. A copy of the report should also be forwarded to the NIH Office of Recombinant DNA Activities (ORDA).**

We will conform to these requirements.

**B. Reports regarding the general progress of patients should be filed with both your local IRB and ORDA within six months of the commencement of the experiment and at six-month intervals thereafter. These twice-yearly reports should continue for a sufficient period of time to allow observation of all major effects. In the event of a patient's death, a summary of the special post mortem studies and statement of the cause of death should be submitted to the IRB and ORDA, if available.**

We will conform to these requirements.

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